Carbohydrate Polymers 3 (1983) 259–272

Aspergillus niger Endopolygalaturonase: 3 — Action Pattern on Polygalacturonic Acid†

Jean-François Thibault

Institut National de la Recherche Agronomique, Centre de Recherches Agro-Alimentaires, Laboratoire de Biochimie et de Technologie des Glucides, 44072 Nantes Cédex, France

(Received: 30 August 1982)

ABSTRACT

The action pattern of an endopolygalacturonase from Aspergillus niger *on polygalacturonate has been investigated by measurements of number- and viscosity-average degree of polymerisation and by gel-permeation. The formation of oligogalacturonate was quantified and compared with theoretical yields. The earlier stage of the degradation (up to 5% hydrolysis) is characterised by a multi-chain attack. Beyond this value, the enzyme proceeds by a non-random attack. Mono-, di- and trigalacturonates are end products with some (4·5% of total galacturonides) resistant material.*

INTRODUCTION

Endopolygalacturonase (poly(1,4 α-D-galacturonide)glycanohydrolase, EC 3.2.1.15) is an important pectic enzyme because of its applications in processing fruit or vegetable products (Rombouts & Pilnik, 1978) and in structural studies of pectic substances (MacNeil *et al.*, 1980; Ishii, 1981). The preceding parts of this series described the purification of an endopolygalacturonase from *Aspergillus niger* (Thibault & Mercier, 1977; Thibault, 1978) and general properties (Thibault & Mercier, 1978). The endo-character of this enzyme was demonstrated by the

† Part of this paper has been presented during the International Symposium on the Use of Enzymes in Food Technology, Versailles, France, May 1982.

Carbohydrate Polymers 0144-8617/83/$03.00 –

fact that a loss of 50% in specific viscosity of polygalacturonate solution was reached when only 2·3% of the α-(1,4)-glycosidic bonds of the substrate were split and by its inability to degrade digalacturonate. The course of hydrolysis of polygalacturonate was studied by thin-layer chromatography. Results showed that mono- to pentagalacturonate are detectable at 29·5% hydrolysis, that pentagalacturonate disappeared at 33% hydrolysis and final products were mono-, di- and trigalacturonate.

This paper presents studies of the action pattern of this enzyme and quantitative aspects of polygalacturonate hydrolysis. The initial stage of the reaction has been studied by changes in viscosity- and number-average degree of polymerisation and by gel-permeation chromatography while oligogalacturonate formation has been followed by gel-permeation chromatography and has been compared with yields calculated from two theories.

MATERIALS AND METHODS

Enzyme

Purified endopolygalacturonase (endoPG) was prepared, from a commercial preparation produced by *Aspergillus niger*, by the method described by Thibault & Mercier (1977).

Substrate

Polygalacturonic acid was purchased from ICN (Cleveland, Ohio, USA). After purification by washing with acid ethanol (1% HCl in 70% ethanol, v/v), the content of anhydrogalacturonic acids was 86·6%, as determined by the *m*-hydroxydiphenyl method (Thibault, 1979). The neutral sugars content, as determined by gas chromatography (Sawardeker *et al.*, 1965; Albersheim *et al.*, 1967) was 7·2% with rhamnose, ribose, arabinose, xylose, mannose, galactose and glucose in the molar ratios: 1/0·1/0·7/1/0·1/3·4/0·3. The polygalacturonic acid contained no methoxyl groups, as determined by the method of Wood & Siddiqui (1971).

Hydrolysis of polygalacturonate by endoPG

The reaction mixture contained substrate (0·2%, w/v), acetate buffer (0·05 M, pH 4·2) and endoPG, at 30°C in a final volume of 75 ml. The

initial action of endoPG was studied after addition of 3 nkatals whilst exhaustive degradation was followed with 11 nkatals. One nkatal is the amount of enzyme which produces one nmole of reducing group, expressed as galacturonic acid, per second. At appropriate intervals, 4 ml of the reaction mixture was pipetted and the enzyme was heat inactivated at 100°C for 10 min. These aliquots were analysed for their specific viscosity with an Ostwald viscometer at 30°C (solvent flow time = 88·9 s) and for their reducing groups by Nelson's method (Nelson, 1944) using galacturonic acid as standard. The viscosity-average degree of polymerisation ($\overline{DP}_v$) was evaluated by the formula given by Smit & Bryant (1967) and the number-average degree of polymerisation ($\overline{DP}_n$) was determined from the reducing end group content. The percentage hydrolysis (h) at any time t was calculated by the equation:

$$h = \frac{\overline{DP}_{n,0} - \overline{DP}_{n,t}}{\overline{DP}_{n,t}(\overline{DP}_{n,0} - 1)} \times 100$$

where $\overline{DP}_{n,0}$ and $\overline{DP}_{n,t}$ were initial $\overline{DP}_n$ and $\overline{DP}_n$ at hydrolysis time t, respectively.

Gel-permeation chromatography

Degraded polygalacturonates were chromatographed on Sephadex G-200 or on Bio-gel P_2, depending on the extent of depolymerisation.

Sephadex G-200 (2·2 × 84 cm) was equilibrated with 0·1 M acetate buffer (ionic strength = 0·1), pH 4. Samples (1·5 ml of the reaction mixture), were injected and the column was eluted in an ascending direction at 17·5 ml h^{-1}. The void volume (V_0 = 118 ml) and total volume (V_t = 300 ml) were determined as the elution volume of commercial pectin and galacturonic acid, respectively. The concentration of galacturonides in the column fractions (3·5 ml) were determined by the *m*-hydroxydiphenyl method (Thibault, 1979). Recoveries from the column varied from 95 to 105%.

Oligogalacturonate formation was followed by gel-permeation chromatography on Bio-gel P_2 as described elsewhere (Thibault, 1980). Aliquots (150–200 µl) of the digests were injected onto the column. The effluent was continuously analysed by the *m*-hydroxydiphenyl method and by the orcinol method (Tollier & Robin, 1979). An aliquot (6 ml/h) of the effluent was introduced into each analytical line. Elution profiles represent the absorbance at 520 nm (*m*-hydroxydiphenyl) and at 425 nm (orcinol) versus elution time, without correc-

tion for the interference of galacturonides during the orcinol detection. Quantitative determination of each oligomer up to heptamer was made by planimetry.

RESULTS AND DISCUSSION

Course of hydrolysis of polygalacturonate by endoPG

The percentage of α-1,4 bonds hydrolysed by the enzyme as a function of reaction time is shown in Fig. 1. The initial degradation rate is constant and high during the first 30 min (hydrolysis = 22%). Beyond this value, reaction rate decreases and becomes zero after 10 h of reaction time (hydrolysis = 53·5%). Further addition of enzyme (5 nkat.) leads to a slight increase in hydrolysis which reaches a final value of 57·5%.

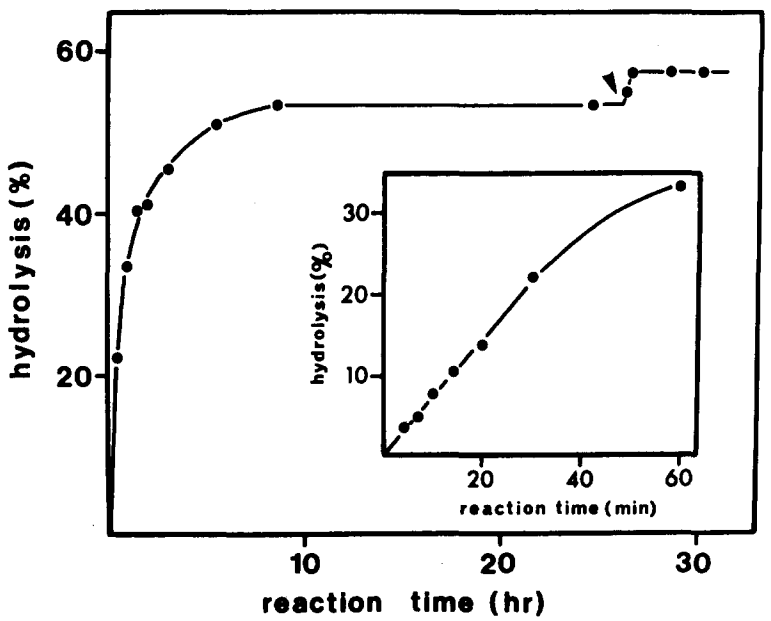

Fig. 1. Hydrolysis of polygalacturonate by endoPG. Reaction mixture contains substrate (0·2%, w/v), acetate buffer (0·05 M, pH 4·2) and enzyme (11 nkat.) at 30°C. Percentage of hydrolysis was measured from the increase of reducing groups. The arrow indicates further addition of enzyme (5 nkat.).

This figure is in agreement with previous works on *Aspergillus niger* endoPG (Thibault & Mercier, 1978) and on *Saccharomyces fragilis* endoPG (Lim *et al.*, 1980).

Study of the initial stage of polygalacturonate hydrolysis

The viscometric technique has been used to study the initial stage of the reaction as this gives a very sensitive measure of the first ruptures of bonds in a polymer. Figure 2 shows that the graph of the reciprocal of $\overline{DP}_v$ versus time is linear up to 25 min (hydrolysis = 4·5%). Vink (1963) demonstrated that such a linear dependence was characteristic of a random hydrolytic process. But just as in the case of the exo-enzymes, several types of action pattern must be considered for endo-enzymes (Banks & Greenwood, 1975): single-chain, multiple attack and multi-chain. The term single-chain implies that after hydrolysing a bond randomly one of the newly-produced chains is completely degraded by the successive removal of small molecules. The term multi-chain refers

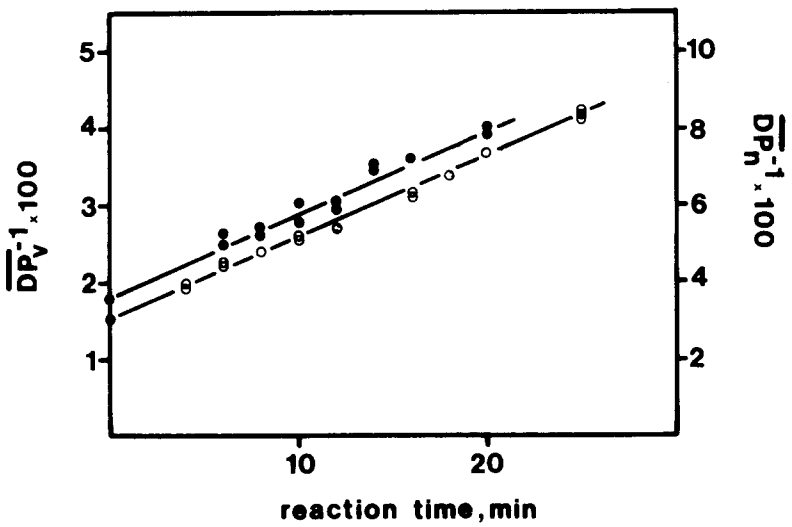

Fig. 2. Graph of the reciprocal of the number-average degree of polymerisation ($\overline{DP}_n$, •) and of the reciprocal of the viscosity-average degree of polymerisation ($\overline{DP}_v$, ○) versus time for the action of endoPG on polygalacturonate. Reaction mixture contains substrate (0·2%, w/v), acetate buffer (0·05 M, pH 4·2) and enzyme (3 nkat.) at 30°C.

to a mechanism in which one bond is hydrolysed randomly in a single enzyme–substrate encounter and multiple attack implies that in addition to the single random rupture, a number of non-random attacks occurs. If an enzyme initially degrades the polymer by multiple-attack or by single-chain, the reaction mixture will contain oligosaccharides of low molecular weight. Viscosity measurements have the disadvantage that they would be unaffected by the presence of low concentrations of such oligosaccharides, which would, however, have an important effect on $\overline{DP}_n$. This is the reason why changes in $\overline{DP}_n$ were also analysed during the initial stage of the hydrolysis. The change in the reciprocal of $\overline{DP}_n$ is linearly related (Fig. 2) to reaction time in the first 20–25 min (corresponding to about 4·5% hydrolysis) so that the ratio $\overline{DP}_v/\overline{DP}_n$ is constant and has a value of 2·2–2·3. This result suggests that, in the initial phase of the degradation (hydrolysis $< 4{\cdot}5\%$), endoPG depolymerises polygalacturonate molecules by a multi-chain mechanism.

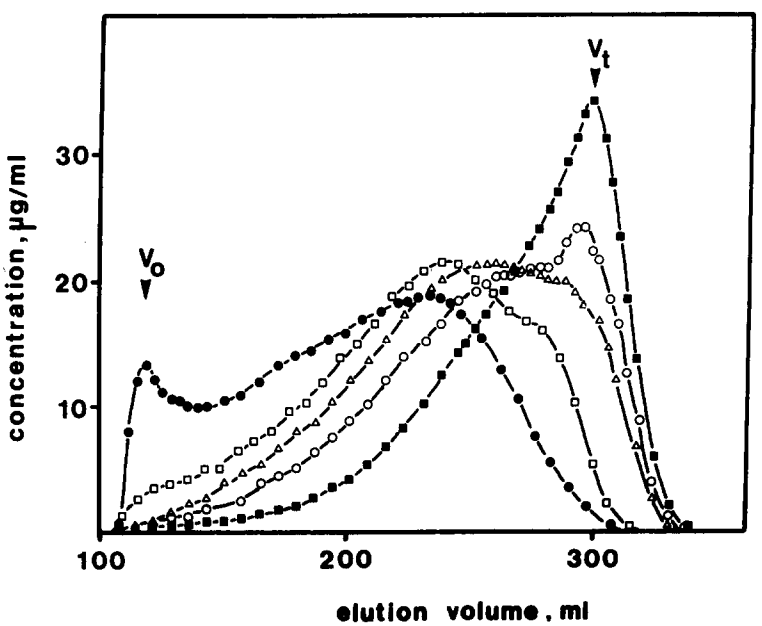

Fig. 3. Gel-permeation chromatography on Sephadex G-200 of polygalacturonate at various extents of hydrolysis. ●, No hydrolysis; □, 33% hydrolysis; △, 4·8% hydrolysis; ○, 7% hydrolysis; ■, 10·5% hydrolysis. V_0 = Void volume; V_t = total volume.

This action pattern was confirmed by gel-permeation chromatography on Sephadex G-200 of the polygalacturonate at different extents of hydrolysis (Fig. 3). The polygalacturonate peak is displaced upon degradation towards the total volume of the column. The fact that this peak does not keep the same elution volume and does not flatten, rules out the possibility of preferential formation of small oligogalacturonides in the initial stage of the reaction. When hydrolysis exceeds 5%, the ratio $\overline{DP}_v/\overline{DP}_n$ is not constant, suggesting another mechanism when the $\overline{DP}$ of the substrate is too low.

The literature is very scarce on this aspect of endoPG action pattern. The endoPG of *Saccharomyces fragilis* apparently shows also a multi-chain action pattern since the enzyme initially degrades polygalacturonate through a series of higher oligogalacturonates (Phaff, 1966). In contrast, the initial products, as well as the final products of polygalacturonate hydrolysis by an endoPG of *Colletotrichum lindemuthianum* (English *et al.*, 1972) are predominantly monogalacturonate and its dimer and trimer. This product formation could be therefore ascribed to a multiple-attack or single-chain action pattern of the enzyme.

Production of oligogalacturonides

In order to confirm that in the later stage of the hydrolysis (> 5%) the enzyme proceeds by a non-random action, quantitative studies on the release of oligogalacturonides have been carried out. The oligogalacturonates produced during hydrolysis were fractionated by gel-permeation chromatography on Bio-gel P_2 and quantified up to the heptamer. Figure 4 shows typical elution patterns. Galacturonate is detectable in the reaction mixture even at 4·5% hydrolysis (not shown in Fig. 4). The degradation of the tetragalacturonate is very slow since it is still present at 53·6% hydrolysis. The final products are mono-, di- and trigalacturonates in a molar ratio of 1/0·6/1·1.

This result confirms our previous work (Thibault & Mercier). The monomer and dimer of galacturonic acid are always end products of polygalacturonate degradation (MacMillan & Sheiman, 1974; Rexova-Benkova & Markovic, 1976) with sometimes the trimer (Saito, 1955; Koller & Neukom, 1969; Ishii & Yokotsuka, 1972). Some workers (Demain & Phaff, 1954; Barash & Eyal, 1970) have observed that the optimum pH for the trimer as a substrate was lower than the optimum

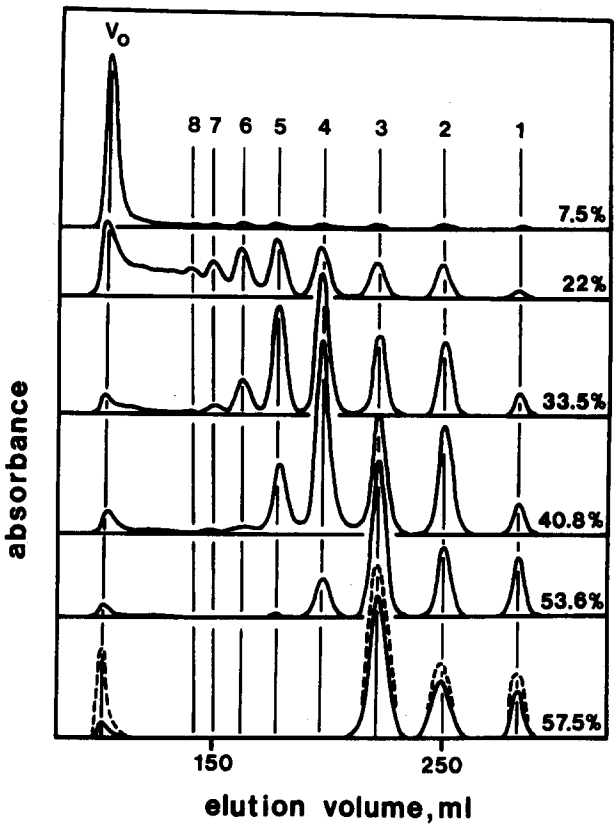

Fig. 4. Gel-permeation chromatography on Bio-gel P_2 of polygalacturonate at various extents of hydrolysis (7·5%, 22%, 33·5%, 40·8%, 53·6% and 57·5%). Numbers 1, ..., 8 represent the degrees of polymerisation of oligogalacturonate; V_0 = void volume. (——) Absorbance at 520 nm (*m*-hydroxydiphenyl detection); (– – –) absorbance at 425 nm (orcinol detection without correction for the interference due to galacturonides).

pH for the polymer. In our case, pure trigalacturonic acid was not hydrolysed by the endoPG in the pH range 2·5–3·8 (phthalate buffer, 0·05 M). This fact does not rule out the inhibitory character of the trimer which has been reported (Demain & Phaff, 1954; Rexova-Benkova, 1973) and denied (Kimura *et al.*, 1973).

In the final stage, 4·5% of total galacturonides are excluded from the gel showing that some material is resistant to the enzyme action.

Detection by the *m*-hydroxydiphenyl (of the galacturonides) and by the orcinol method (of neutral and acidic sugars) was achieved. For the peaks corresponding to mono-, di- and trimer, the ratio between the area obtained from the galacturonides detection and the area obtained from the orcinol detection was constant and equal to that obtained from a standard galacturonate under the same conditions. This result shows that these peaks correspond to true oligogalacturonates. In contrast, the material eluted at the void volume of the column contains all the neutral sugars and some galacturonic acids in a molar ratio (calculated for hexoses) of 2/1. The presence of such a material suggests a blockwise distribution of neutral sugars as side-chains as well as rhamnose 'kinks'.

The experimental product distribution curve in the hydrolysis of polygalacturonate catalysed by endoPG is shown in Fig. 5. It can be seen that tetragalacturonate is cleaved into mono- and trigalacturonate,

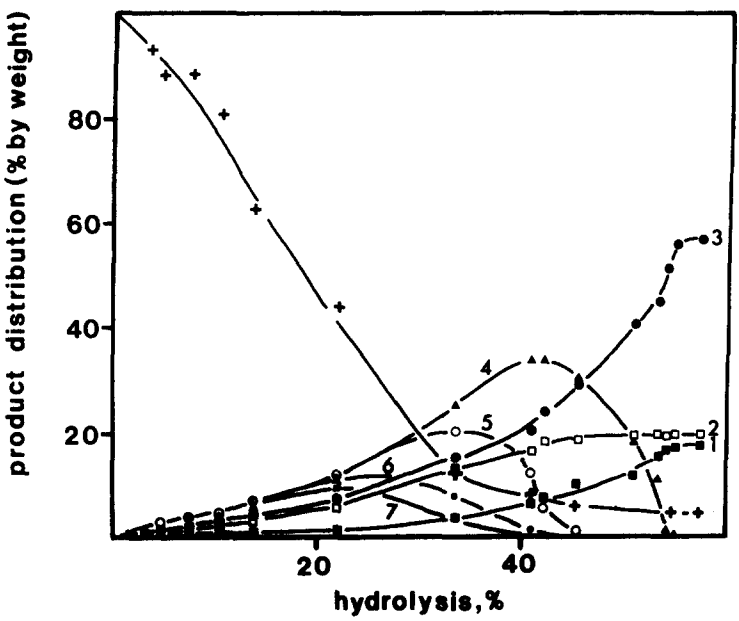

Fig. 5. Experimental product distribution curves in the hydrolysis of polygalacturonate. Numbers represent the degree of polymerisation of oligogalacturonates; the symbol + is used to represent the material excluded from the Bio-gel P_2 column.

as digalacturonate content does not change after 40% hydrolysis while higher oligogalacturonides are characterised by several modes of cleavage:

$$GA_4^{\dagger} \longrightarrow GA_3 + GA_1$$

$$GA_5 \begin{cases} \longrightarrow GA_4 + GA_1 \\ \longrightarrow GA_3 + GA_2 \end{cases}$$

$$GA_6 \begin{cases} \longrightarrow GA_5 + GA_1 \\ \longrightarrow GA_4 + GA_2 \\ \longrightarrow GA_3 + GA_3 \end{cases}$$

This pattern is in accord with the mode of action of endoPG from *Aspergillus* sp., as described by Rexova-Benkova & Markovic (1976).

Earlier stages are characterised by the presence of higher oligogalacturonides since at 20% hydrolysis the main products are hepta-, hexa- and pentamers. Koller & Neukom (1969) observed the accumulation of tri- and hexagalacturonates as the preponderant products of the hydrolysis of polygalacturonate and, as a conclusion, suggested that the binding groups of substrate are separated from the catalytic centre by a distance of three and six galacturonate units, respectively. Our experiment does not confirm this interpretation. Very little information is available in the literature concerning the quantitative aspects of the polygalacturonate hydrolysis. Liu & Luh (1980) studied the degradation of polygalacturonate by endoPG from *Rhizopus arrhizus* by thin-layer chromatography. The end product was monogalacturonate, suggesting the presence of an exo-PG activity. MacClendon (1979) determined by gel-permeation chromatography on Sephadex G-25 the amounts of the oligomers produced in hydrolysis of polygalacrutonate by *Saccharomyces fragilis* endoPG. His results are difficult to compare with ours because hydrolysis values are not available but apparently, endoPG from *Saccharomyces fragilis* produces more trigalacturonate and less mono- or digalacturonate than endoPG from *Aspergillus niger*.

In Table 1, the experimental yields of oligogalacturonates were compared to theoretical yields. If the degradation is random, the weight fraction of i-mer, $w(i)$, is (Painter, 1963):

$$w(i) = \frac{i}{\overline{DP}_0} [2s(1-s)^{i-1} + (\overline{DP}_0 - s - 1)\, s^2(1-s)^{i-1}] \qquad (1)$$

† GA_n = Oligogalacturonic acid; n = degree of polymerisation.

TABLE 1

Experimental and Theoretical Yields of Oligogalacturonates Produced from the Action of endoPG on Polygalacturonate

	Hydrolysis (%)	*Oligogalacturonate[a] (% by wt)*							
		GA_1	GA_2	GA_3	GA_4	GA_5	GA_6	GA_7	*Higher oligomers*
Experiment	4·8	0·3	1·0	1·7	1·9	2·4	3·4	← 89·3 →	
Random[b]		0·5	1·0	1·4	1·8	2·1	2·4	← 90·8 →	
GA_6^a stable[c]		0·5	1·0	1·5	2·0	2·5	3·0	← 89·5 →	
Experiment	13·8	0·9	3·6	4·5	6·1	7·9	7·7	6·9	62·4
Random		2·7	4·6	5·8	6·5	6·8	6·8	6·7	60·1
GA_5 stable		1·5	3·1	4·6	6·1	7·7	← 77 →		
Experiment	33·5	3·7	13·4	15·2	25·5	20·5	7·9	3·1	10·7
Random		12·8	16·5	15·9	13·6	11·0	8·4	6·3	15·5
GA_4 stable		5·7	11·6	17·3	23·1	← 42·3 →			
Experiment	42·0	7·9	18·5	24·5	35·1	5·3	0·6	0	8·1
Random		17·9	20·7	17·9	13·8	10·0	6·9	4·6	8·2
GA_4 stable		8·6	17·2	25·7	34·3	← 14·2 →			
Experiment	57·1	18·5	20·1	57·2	0	0	0	0	4·2
Random		34·9	28·6	17·6	9·6	4·9	2·4	1·1	0·9
GA_3 stable		15·9	31·8	47·8	← 4·5 →				

[a] GA_n = oligogalacturonate, n = degree of polymerisation.

[b] Calculated from eqn. (1), see Results and Discussion section.

[c] Calculated from eqn. (2), see Results and Discussion section; $i = 6, 5, 4, 3$.

where s is the degree of scission ($s = h/100$) and $\overline{DP}_0$ is the average degree of polymerisation of the substrate, taken as the number-average. Table 1 shows that this theory is in good agreement with the experimental values if hydrolysis does not exceed 5%. This fact confirms the random hydrolytic process in the earlier stage of the degradation. Beyond this value, this theory is not suitable, as a statistical product distribution was obtained and much more monomer was observed. If the j-mer is resistant to further hydrolysis, Painter (1963) and Greenwood *et al.* (1965*a*) demonstrated that the yield in $(j-i)$-mer can be calculated by

$$w(j-i) = \frac{2(j-i)}{j(j+1)}\alpha \qquad i = 0 \text{ to } (j-1) \tag{2}$$

In our experiments, the reaction mixture was analysed before complete degradation and results must be corrected with the fraction (α) of polymer which has been degraded (Greenwood *et al.*, 1965*a*, *b*).

This theory accounts quite adequately for the observed yields up to 42% hydrolysis. The oligomer resistant to hydrolysis was taken as the most abundant one in the reaction mixture. As the degradation increases, the degree of polymerisation of the resistant oligomer decreases, suggesting that endoPG attacks higher oligogalacturonates more readily. But the final stage of the reaction does not follow such a pattern since this theory gives lower amounts in tri- and monogalacturonate and higher amounts in digalacturonate than observed. The non-degradability of the trimer could explain this disagreement.

Hence, the hydrolysis of polygalacturonate by *Aspergillus niger* endoPG is characterised by two apparent stages. In the earlier stage (up to 5% hydrolysis) the degradation involved a multi-chain attack, i.e. all the bonds of the substrate have the same probability of scission. When the degree of polymerisation of the substrate decreases, end-effects involve a very slow and a non-random degradation. Similar features have been found for α-amylases (Greenwood *et al.*, 1965*b*; Greenwood & Milne, 1968). The second stage could be explained on the basis of two theories; namely, those of multiple-attack and preferred attack, as described by Banks & Greenwood (1977) for α-amylases.

ACKNOWLEDGEMENT

The author is indebted to Mrs M. Chebrout for excellent technical assistance.

REFERENCES

Albersheim, P., Nevins, D. J., English, P. D. & Karr, A. (1967). *Carbohydr. Res.* **5**, 340.

Banks, W. & Greenwood, C. T. (1975). *Starch and its components*, Edinburgh University Press, Edinburgh, p. 211.

Banks, W. & Greenwood, C. T. (1977). *Carbohydr. Res.* **57**, 301.

Barash, I. & Eyal, Z. (1970). *Phytopathol.* **60**, 27.

Demain, A. L. & Phaff, H. J. (1954). *J. Biol. Chem.* **210**, 381.

English, P. D., Maglothin, A., Keegstra, K. & Albersheim, P. (1972). *Plant Physiol.* **49**, 293.

Greenwood, C. T. & Milne, E. A. (1968). *Stärke* **4**, 101.

Greenwood, C. T., MacGregor, A. W. & Milne, E. A. (1965*a*). *Arch. Biochem. Biophys.* **112**, 466.

Greenwood, C. T., MacGregor, A. W. & Milne, E. A. (1965*b*). *Carbohydr. Res.* **1**, 303.

Ishii, S. (1981). *Phytochem.* **20**, 2329.

Ishii, S. & Yokotsuka, T. (1972). *Agr. Biol. Chem.* **36**, 1885.

Kimura, H., Uchino, F. & Mizushima, S. (1973). *J. Gen. Microbiol.* **74**, 127.

Koller, A. & Neukom, H. (1969). *Eur. J. Biochem.* **7**, 485.

Lim, J., Yamasaki, Y., Suzuki, Y. & Ozawa, J. (1980). *Agric. Biol. Chem.* **44**, 473.

Liu, Y. K. & Luh, B. S. (1980). *J. Food Sci.* **45**, 601.

MacClendon, J. H. (1979). *Phytochem.* **18**, 765.

MacMillan, J. D. & Sheiman, M. I. (1974). Pectic enzymes. In: *Food related enzymes*, ed. J. R. Whitaker, Am. Chem. Soc., Washington, DC, p. 101.

MacNeil, M., Darvill, A. G. & Albersheim, P. (1980). *Plant Physiol.* **66**, 1128.

Nelson, N. (1944). *J. Biol. Chem.* **153**, 375.

Painter, T. J. (1963). *J. Chem. Soc.* **145**, 779.

Phaff, H. J. (1966). *Methods in Enzymology* **8**, 636.

Rexova-Benkova, L. (1973). *Eur. J. Biochem.* **39**, 109.

Rexova-Benkova, L. & Markovic, O. (1976). Pectic enzymes. In: *Adv. carb. chem. biochem.*, Vol. 33, eds. R. S. Tipson and D. Horton, Academic Press, New York, p. 323.

Rombouts, F. M. & Pilnik, W. (1978). *Process Biochem.* **13**, 9.

Saito, H. (1955). *J. Gen. Appl. Microbiol.* **1**, 38.
Sawardeker, J. S., Sloneker, J. H. & Jeanes, A. (1965). *Analyt. Chem.* **37**, 1602.
Smit, C. J. B. & Bryant, E. F. (1967). *J. Food Sci.* **32**, 197.
Thibault, J. F. (1978). *C. R. Acad. Sci.* (D) **287**, 1433.
Thibault, J. F. (1979). *Lebensm. Wiss. u. Technol.* **12**, 247.
Thibault, J. F. (1980). *J. of Chromatogr.* **194**, 315.
Thibault, J. F. & Mercier, C. (1977). *J. Solid-Phase Biochem.* **2**, 295.
Thibault, J. F. & Mercier, C. (1978). *J. Food Biochem.* **2**, 379.
Tollier, M. T. & Robin, J. P. (1979). *Ann. Technol. Agric.* **28**, 1.
Vink, H. (1963). *Makromol. Chem.* **67**, 105.
Wood, P. J. & Siddiqui, I. R. (1971). *Analyt. Biochem.* **39**, 418.