The Chromatographic Examination of the Products of the Action of Pectinase on Pectin

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A large number of bacteria and fungi secrete extracellularly the enzyme complex often termed 'pectinase', which causes the rapid disintegration of plant tissue and is considered the primary instrument of the attack on plant tissues by these micro-organisms. The action of pectinase on a solution of pectin results in the following changes: (*a*) a rapid lowering of the viscosity of the solution; (*b*) a failure of the solution to form a gel on the addition of ethanol or solutions of calcium salts, only a gelatinous precipitate or a cloudy suspension being formed; (*c*) an increase in the titratable acidity of the solution; (*d*) an increase in the reducing power of the solution, as measured by the iodine-reducing value.

These effects are now considered to be due to the action of two distinct enzymes: 'pectin esterase' and 'polygalacturonase'. The increase in acidity is due to the demethylation of the pectin by pectin esterase (Holden, 1948). The lowering of viscosity, failure of gelation, and increase in reducing power are due to the degradation of pectin by polygalacturonase which has been claimed (Jansen & MacDonnell, 1945) to act only very slowly on true pectin but rapidly on demethylated pectin (pectic acid). Changes in viscosity and gelling properties are brought about fairly rapidly even by very weak enzyme preparations, but marked changes in reducing power only appear with active preparations.

The mechanism of the demethylation of pectin to pectic acid and the action of polygalacturonase have been extensively investigated and the literature has been reviewed by Phaff & Joslyn (1947).

The main object of the present investigation has been to study by chromatographic methods the products arising from the degradation of pectic acid by polygalacturonase.

MATERIALS AND METHODS

Preparation of pectinase

The pectinase preparations used in this study were made from dried bran cultures of the following moulds: *Botrytis cinerea*, *Penicillium expansum* and *Aspergillus aureus*. Samples (30 g.) of the cultures were extracted with 300 ml. of distilled water adjusted to pH 5·6 by adding N-HCl. The enzyme was precipitated either by the addition of 5 vol. of 95% (v/v) aqueous ethanol or 70 g. $(NH_4)_2SO_4$/100 ml. of extract.

The precipitate obtained on addition of ethanol was collected by filtering through a layer of Kieselguhr and a Whatman no. 41 filter paper on a Büchner funnel, and then taken up in 40 ml. water and again filtered. The precipitate obtained by the addition of $(NH_4)_2SO_4$ was also filtered through Kieselguhr and a no. 41 paper. It was transferred with the Kieselguhr and a minimum quantity of water to a dialysing sac and dialysed overnight against tap water to remove the $(NH_4)_2SO_4$. The sac contents were then filtered, made up to 40 ml. and used directly, since attempts to prepare an active dried preparation were not successful. Of these two methods, precipitation with $(NH_4)_2SO_4$ usually gave the more active preparation.

Purification of pectin

Pectin was purified by extracting 50 g. of commercial 100-grade citrus pectin twice with 500 ml. portions of 70% (v/v) aqueous ethanol for 20 min. and drying the residue at 37°. The resulting samples had the following characteristics as determined by methods recommended by Hinton (1939): saponification value, 8·95; free acidity, 3·5; iodine-reducing value, 0·5.

Preparation of low-methoxy pectin

Low-methoxy pectin was prepared by stirring 100 g. of commercial pectin with a mixture of 375 ml. of 95% (v/v) aqueous ethanol and 125 ml. of N-NaOH twice for 20 min. and then washing with a mixture of 200 ml. of 95% ethanol, 20 ml. of water and 10 ml. of N-HCl and finally drying at 37°.

The product had the following characteristics: saponification value, 0·25; free acidity, 1·8; iodine-reducing value, 0·6. It was thus mainly the Na salt of an almost completely demethylated pectic acid, in which not all the carboxyl groups had been neutralized.

The action of pectinase on pectin

A 1% (w/v) solution of pectin was prepared. Since the salt concentration produced by the addition of sufficient buffer solution to maintain a constant pH would have caused difficulties in separating the products on the paper chromatogram, the pH was adjusted initially to 6·0 (using chlorophenol red as indicator) by adding small amounts of N-HCl or N-NaOH. During the subsequent action of pectinase, low-methoxy pectin solutions required little or no pH adjustment, since few additional free carboxyl

groups were liberated; the pH of the pectin solutions was maintained by the addition of small amounts of N-NaOH. A sample of 250 ml. of the 1% pectin was taken, and 17 ml. were withdrawn initially for the following tests: 10 ml. for the determination of iodine-reducing values; 1 ml. for a gelling test with ethanol; 1 ml. for a gelling test with $CaCl_2$; 5 ml. transferred to a test tube, heated immediately in a boiling-water bath for 5–10 min. to inactivate the enzyme and then stored with the addition of a drop of $CHCl_3$-toluene mixture (1:3 (v/v)) at 0° for chromatographic examination at a later date.

To the remaining 233 ml. were added 40 ml. of enzyme solution, giving a final pectin concentration of 0·85% (w/v). The enzyme-pectin mixture was incubated at 20°, and 17 ml. samples were withdrawn at convenient intervals to be tested in the same manner as the initial enzyme-free solution.

Iodine-reducing value

The solution (10 ml.) was added to 50 ml. of water, followed by 3 ml. of N-NaOH and 20 ml. of 0·1 N-I_2 solution. After 10 min. the solution was acidified with 10 ml. of N-HCl, and the remaining iodine was titrated with 0·1 N-sodium thiosulphate solution. A blank titration without pectin was carried out at the same time, and the difference in titre gave the amount of 0·1 N-I_2 solution reduced by 10 ml. of pectin solution (see Hinton, 1939).

Gelling tests with ethanol and calcium salts

To 1 ml. samples of the pectin solution were added either (*a*) 5 ml. of 95% (v/v) aqueous ethanol or (*b*) 1 ml. of 1% (w/v) $CaCl_2$ solution. Gelation with Ca ions depends on the presence of free carboxyl groups and fails with methylated pectin; the test was therefore applied to low-methoxy pectins only. The precipitates formed were compared with standards selected from a series obtained by allowing pectinase to act on a pectin solution for increasing periods of time. These standards were graded as follows: (1) rigid gel; (2) soft gel, disintegrating on shaking; (3) curdy gel; (4) heavy gelatinous precipitate; (5) slight gelatinous precipitate; (6) flocculent dispersal.

Chromatographic tests

It was found that the most satisfactory solvent system for separating the galacturonic acid oligosaccharides on the paper chromatogram was *iso*butyric acid-water; being near its critical point it gives high R_F values for monosaccharides (Jermyn & Isherwood, 1949), and thus allows a reasonable space on the paper chromatogram behind galacturonic acid for the separation of the galacturonic acid oligosaccharides. Since *iso*butyric acid is an acid solvent these acid sugars travel normally on the paper chromatogram without the distortions due to ionic effects found with neutral and basic solvents.

The paper used was Whatman no. 541, which gave rapid solvent flow. No distortion of the shape of uronic acid spots due to the heavy-metal ions present in such paper as Whatman no. 1 was observed, and the uronic acids appeared on development as nearly circular spots and not as elliptical or multiple spots (cf. the experiences of Hanes & Isherwood (1949) with the development of phosphoric esters on paper chromatograms).

The following technique was used. Equal volumes (7 μl.) were withdrawn by micropipette from each of the 5 ml. samples taken during the course of hydrolysis and delivered to the appropriate position on the base line of a piece of paper of suitable size prepared for use as a paper chromatogram. The line of equal-sized spots formed a time series from the beginning to the end of the hydrolysis. The chromatogram was developed in the apparatus described by Jermyn & Isherwood (1949), using the precautions outlined by them. The solvent was allowed to drip from the end of the 40 cm. paper and the total time of running was 20 hr. The fact that the acids were initially present as the Na salts did not appear to have any effect on their separation; spots applied as galacturonic acid and sodium galacturonate were found to travel at exactly the same rate. After drying off the solvent, the positions of the various sugar spots on the paper were revealed by spraying with ammoniacal silver nitrate and heating at 105°. Prolonged heating (20–25 min.) was necessary to detect the compounds of higher molecular weight, and it is probable that the reaction resembled that described by Partridge (1948) for such polyhydroxy compounds as inositol, especially as the more specific sugar reagents were quite ineffective.

Measurement of viscosity

Changes in relative viscosity were followed by measuring the time taken for the pectin solution to run from a 25 ml. pipette, the tip of which had been removed and replaced by a piece of capillary glass tubing 16·2 cm. in length and 0·123 cm. in diameter. The time taken for water to run from the pipette was 25 sec.; a 1% (w/v) solution of commercial 100-grade citrus pectin took 100–150 sec. and 1% solution of purified pectin took 300–400 sec.

RESULTS

Iodine-reducing values

Fig. 1 shows changes in iodine-reducing value obtained with an active and a less active preparation of *P. expansum* acting on low-methoxy pectin.

Fig. 2 shows changes in iodine-reducing value obtained with active preparations from *A. aureus*, *P. expansum* and *B. cinerea* acting on low-methoxy pectin.

On comparing the action of preparations from *B. cinerea* on purified pectin and on low-methoxy pectin (Fig. 3), similar results were obtained. This indicates that the enzyme preparation was rich in pectin esterase as well as in polygalacturonase.

When a pectin (fully methylated pectic acid) of very great chain length is completely degraded to galacturonic acid, the iodine-reducing value of a 0·85% (w/v) solution ought to rise from zero to 8·95 and for the 0·85% sodium pectate solution, to which the low-methoxy pectin solutions used in this study approximate, the rise would be from zero to 8·6. Fig. 2 shows that an increase in iodine-reducing value is obtained that approaches these figures. The rise in iodine-reducing value at any stage of the enzymic hydrolysis ought thus to provide a basis for calculating the percentage of glycosidic linkages

which have been broken. This calculation has not been made for the following reasons:

(*a*) The pectin preparations have a definite reducing value (0·5–0·6) which can be only slowly

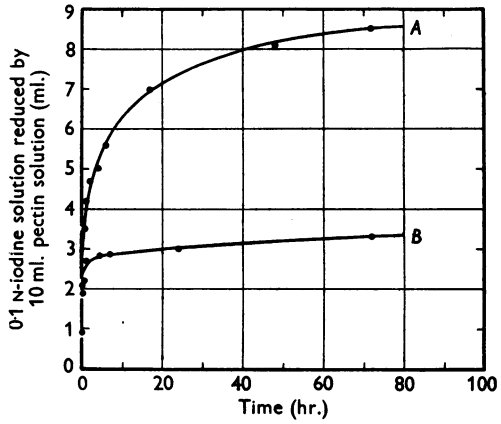

Fig. 1. Change in iodine-reducing value produced by the action of an active (*A*) and by a less active (*B*) pectinase preparation from *P. expansum* on low-methoxy pectin.

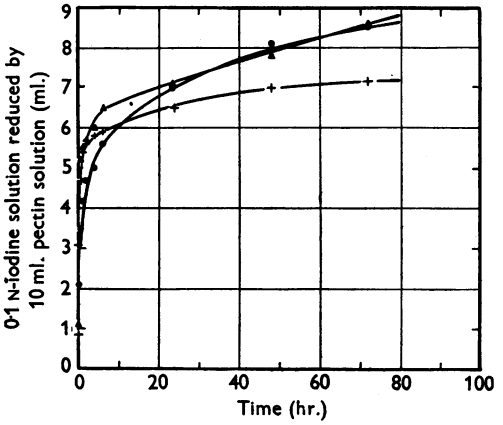

Fig. 2. Change in iodine-reducing value produced by the action of pectinase from *B. cinerea*, *P. expansum* and *A. aureus*. ▲, *B. cinerea*; ●, *P. expansum*; +, *A. aureus*.

lowered by further washing; it is partly due to retained sugar (glucose) and partly to short-chain polysaccharides such as arabans.

(*b*) The enzyme preparations themselves have a significant reducing value.

(*c*) The pectin preparations are not simply long-chain polygalacturonides; other ('ballast') polysaccharides are present to an uncertain extent. Hence a nominally 0·85% (w/v) pectin solution actually contains less than 0·85% of polygalacturonide; furthermore, it is uncertain to what extent sugars formed from these other polysaccharides will add to the observed iodine values.

Complete hydrolysis of pectin by chemical methods might be expected to give an independent check which would avoid some of the uncertainties inherent in the enzymic method. The yield of galacturonic acid from acid hydrolysis under various conditions was compared by means of quantitative paper chromatography (Jermyn & Isherwood, 1949) with that from hydrolysis with *B. cinerea* preparations. Even the most satisfactory complete acid hydrolysis (boiling with 3% (w/v) nitric acid for 12 hr.) gave only 89% of the final yield from the enzymic hydrolysis. Hydrolysis was in all cases judged to be complete when no galacturonic acid oligosaccharides were visible on a qualitative paper chromatogram.

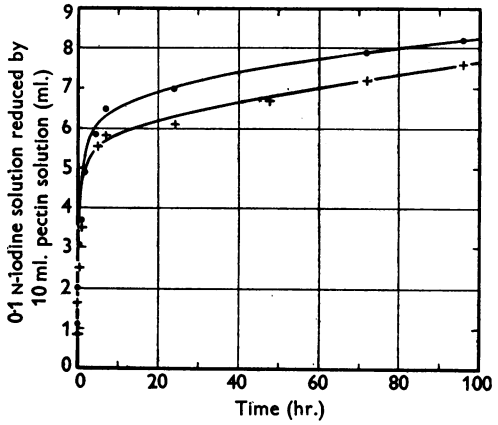

Fig. 3. Change in iodine-reducing value of pectin and of low-methoxy pectin produced by the action of enzyme preparation from *B. cinerea*. +, Low-methoxy pectin; ●, pectin.

In the absence of any accurate basis of comparison, such as might eventually be provided by quantitative paper chromatography, no attempt was made to convert the data for iodine reduction shown in Figs. 1–3 to exact values for percentage scission in any given case; though rough estimates (±5%) could be made for purposes of comparison.

Chromatographic observations

With all the enzyme preparations tested, and using both pectin and low-methoxy pectin, the following effects were observed on the paper chromatogram:

(*a*) A series of up to five spots appeared on development of the chromatogram. In addition there was, in the early stages of the hydrolysis, a short initial streak running from the base-line position down to the slowest moving spot.

(*b*) The fastest moving spot could be identified by direct comparison as being due to galacturonic acid. It appeared somewhat earlier than the slower moving substances and increased steadily in size and depth of colour till the end of the hydrolysis, when it was the sole remaining spot. There often appeared, in front of the galacturonic acid spot, spots due to various sugars, glucose (constant in size during the hydrolysis and due to initial contamination of the pectin), galactose and arabinose. The two last were before the first sample (generally 30 min. after the start of the experiments) was taken.

These observations are illustrated by Fig. 4, which shows the stages in the hydrolysis of low-methoxy pectin by an enzyme preparation from *B. cinerea*.

No attempt was made to apply quantitative chromatographic methods to the mixtures of compounds occurring at various stages of the hydrolysis, since the pure compounds *A*, *B*, *C* and *D* were not

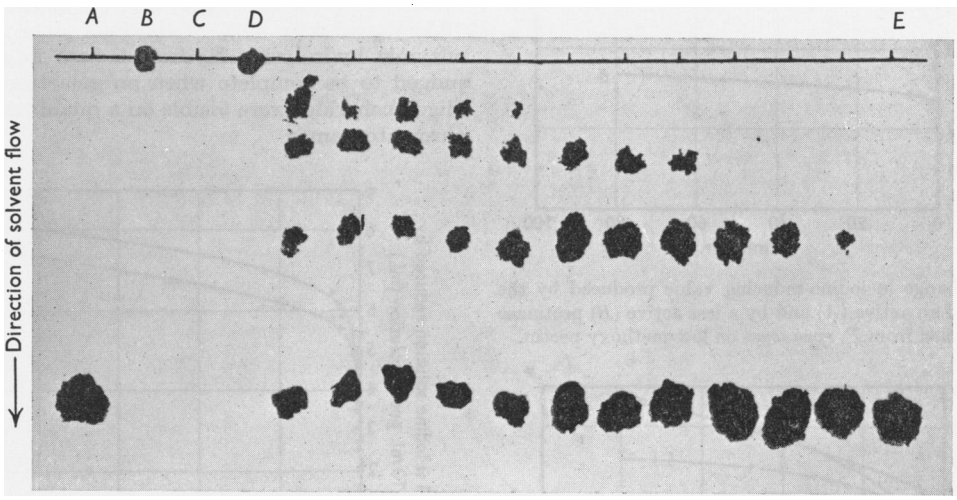

Fig. 4. Changes in the composition of a solution of low-methoxy pectin acted upon by an active preparation of pectinase from *B. cinerea*. *A*, marker spot of galacturonic acid; *B*, pectin solution alone (5 parts + 1 part water); *C*, enzyme solution alone (1 part + 5 parts water); *D–E*, mixture of pectin and enzyme solutions (5:1) at various intervals of time: 0·0, 0·25, 0·5, 1, 1·5, 2·5, 4, 6, 8, 24, 48, 73, 96 hr. This figure has been prepared from a reflex print of a chromatogram run at 20° for 20 hr. on Whatman no. 541 paper, with *iso*butyric acid as the developing solvent (see text, p. 438).

erratic in appearance as between different runs, and their amount obviously depended on the amount of the ballast polysaccharides, galactan and araban, in the pectin samples and the degree to which galactanases and arabanases occurred in the crude enzyme preparations.

(*c*) The slower moving spots all waxed in size and colour and then waned and disappeared as hydrolysis proceeded. The slower a spot moved, the sooner it waxed and waned. The two faster moving spots of this group (corresponding to compounds *A* and *B*) were present up to a late stage in all chromatograms examined. The next, corresponding to compound *C*, was present for a considerable time with the less active preparations, but with increasingly active preparations it waxed and waned progressively earlier in the process of hydrolysis. The slowest spot, corresponding to substance *D*, only appeared with the less active preparations; with very active preparations it had reached a maximum and disappeared

available for comparison. It was shown that, using a powdered cellulose column and the partition chromatogram technique of Martin & Synge (1941) with the two phases of the system *iso*butyric acid-water, it was possible to obtain fractions of the effluent containing only *A*, *B* or *C* in solution. Since the effect of these substances on the phase equilibrium of the nearly critical *iso*butyric acid-water system was extremely marked, it was necessary to work at such low total concentrations of solute (about 5 mg./100 ml.) to obtain satisfactory resolution on the column that quantities sufficient for isolation and identification were not obtained.

The R_F values of the various compounds for the *iso*butyric acid-water system were: galacturonic acid, 0·295; substance *A*, 0·16; *B*, 0·09; *C*, 0·055; *D*, 0·035 (approx.).

The R_F values are very sensitive to the experimental conditions employed, and that of galacturonic acid can vary between 0·2 and 0·5 unless

particular attention is paid to temperature control and equilibration (Jermyn & Isherwood, 1949).

Correlation of results: gelling and viscosity data

Although the reactions between the various enzyme and pectin preparations took place at different rates, it appeared that the degradation of pectin by the polygalacturonase moiety of the 'pectinase' complex could be correlated with the degree of bond scission as measured by the iodine-reducing value and with observations on the paper chromatogram. Experiments with enzyme preparations of low activity showed the effects on the properties of pectin of the scission of a small percentage of its glycosidic bonds; the course of events over the complete range could then be deduced by comparison with the results from experiments using active preparations in which the earlier events took place in less than 30 min. The results of experiments with preparations of intermediate activity showed many of the features observed with both low- and high-activity preparations, but failed to give the results observed at the extreme ends of the range.

(*a*) Viscosity fell rapidly before any noticeable rise in iodine-reducing value occurred. The viscosity of the solution was reduced to a value scarcely exceeding that of water before 10% scission had taken place.

(*b*) With low-methoxy pectin the degree of gelation on addition of calcium chloride fell from grade 1 to grade 4 when 20% of scission had taken place and to below grade 6 (i.e. no visible opalescence) at 30% scission.

(*c*) On addition of ethanol the degree of gelation fell from grade 1 to grade 4 in the range up to 30% scission, and from grade 4 to grade 6 in the range 30–50%. There was always a slight flocculent precipitate, which was observable even with a solution of pure sodium galacturonate of the appropriate concentration.

(*d*) Compound *D* appeared on the paper chromatogram before 30% scission, and had disappeared by the time scission had reached 60%. Compound *C* appeared in the range 30–70%, with a maximum at 45%. Compound *B* occupied the range 30–95%, with a maximum at 65%. Compound *A* occupied the range 30–100%, with a maximum at 75%. Galacturonic acid was first definitely visible on the paper chromatogram when scission was about 25% complete, and thereafter rose steadily in amount until it was finally the only degradation product visible.

DISCUSSION

It is known that 'pectin' is essentially a chain of α-1:4-linked galacturonic acid residues esterified with methanol, and that the typical properties of pectin depend on the integrity of this chain. Certain other polysaccharides are always associated with it in preparations which have not been rigorously purified, but it is believed (Hirst & Jones, 1946) that these polysaccharides are not part of the pectin structure. The degradation of pectin by 'pectinase' is thus essentially the splitting of the linkages of polygalacturonic acid by polygalacturonase.

An enzyme hydrolysing a polysaccharide chain might conceivably act in three ways: (1) by removing single sugar units from the ends of the chains; (2) by removing groups of units from the ends of chains, followed by the hydrolysis of these oligosaccharides by a second enzyme or (3) by causing random scission of the chains, leading eventually to single sugar units as in the action of α-amylase on the unbranched chain of the amylose components of starch.

The hydrolysis of pectic acid by polygalacturonase seems to follow the third of these courses. To give a distinct spot on the paper chromatogram any fragment of the polygalacturonide chain resulting from the hydrolysis must be of sufficiently low molecular weight to move under the conditions employed and be present in sufficient concentration to react visibly with the colouring reagent. In the case of random scission these conditions would not be fulfilled until a considerable proportion of the bonds had been broken, which conforms with the present observations. Each of the lower oligosaccharides would be expected to show a maximum and then slowly to disappear from the paper chromatogram; the maximum and point of invisibility being the earlier the more complex the oligosaccharide. This again conforms with the present observations.

The regularity of the successive R_F values of galacturonic acid and the compounds designated *A*, *B*, *C* and *D* indicates that these compounds form a series of regularly increasing molecular complexity. This regularity is even more striking when the R_M ($\log [1/R_F - 1]$) values of the series are calculated (Bate-Smith & Westall, 1950; cf. Brønsted, 1931, 1938; Brønsted & Warming, 1931). These increase in regular steps, the values being (galacturonic acid first) 0·38, 0·72, 1·01, 1·23, 1·43. The assignment of the digalacturonic acid structure to compound *A* is confirmed by its R_F value (0·16) being almost identical with that of cellobiose (0·155); α-1:4-digalacturonic acid and cellobiose are very close structural analogues. Hence the series disclosed by the chromatographic data may, with some justification, be identified as mono-, di-, tri-, tetra- and penta-galacturonic acids, and it is on this assumption that the experimental results of this study have been interpreted. This is the series to be expected from the random scission of polygalacturonic acid chains, and a similar series is known from the acid hydrolysis of cellulose (Zechmeister & Tóth, 1931), which has been shown statistically to be a process of random scission. Any other regular series (e.g. di-,

tetra-, hexa-, etc.) would imply that polygalacturonase is a complex of enzymes differing in specificities. No proof of the postulated allocation of structure is provided here, and other possibilities cannot, therefore, be absolutely excluded on the present data.

The observed rapid fall in the rate of the reaction with time conforms with what would be expected for the reaction of a single enzyme with a decreasing population of glycosidic linkages. Since this study is a preliminary one and is concerned with what are most probably heterogeneous substrates and enzymic systems, no kinetic analysis has been attempted.

The typical properties of pectin solutions are apparently associated with molecules of chain length considerably greater than five residues; gelation with ethanol and calcium ions fails before any considerable proportion of chains of short length is detectable by the paper chromatogram. Viscous solutions are apparently associated with very considerable chain length. Jansen, MacDonnell & Ward (1949) have shown that a 'pectin' of average chain length 32 still retains most of the recognized properties of pectin. Those properties of the polygalacturonide chain which characterize 'pectin' therefore disappear between the limits of 32 (average) and 5 units of chain length, and the results reported in this study show that they do not disappear simultaneously, e.g. the capacity for forming a 'calcium pectate' gel disappears before that for forming a gel with ethanol.

SUMMARY

1. Polygalacturonase preparations from moulds hydrolyse pectic acid by random scission of the glycosidic linkages of the polygalacturonide chain. When the chain length of the resultant molecular fragments is 5 or less, they move appreciably on a paper chromatogram developed with *iso*butyric acid.

2. The final stages of the hydrolysis, after the iodine-reducing values show that about 30 % of the glycosidic linkages have been broken, can be studied on the paper chromatogram by observing the relative concentrations of these fragments at various stages during the hydrolysis.

3. The later stages of this hydrolysis are the same with 'pectinase' from three different moulds acting on 'pectin' or 'low-methoxy pectin'; the initial rates vary with the enzyme source and the degree of methylation of the pectin.

4. The rate of hydrolysis becomes very slow after about 50 % of the linkages have been broken; with enzyme preparations of low activity it is so slow that complete scission has not been attained.

5. The typical properties of 'pectin'—calcium gelation, ethanol gelation, highly viscous solutions—disappear before molecular fragments embodying 5 or fewer residues of galacturonic acid appear on the paper chromatogram.

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