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Polygalacturonase in Normal and Abnormal Tomato Fruit

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The pectic enzymes are widespread in the plant kingdom and their complexity is becoming more obvious (Demain & Phaff, 1957; Deuel & Stutz, 1958). They are assumed to play an intimate part in fruit ripening (Ulrich, 1957; Biale, 1960) and are essentially hydrolases. After the solubilization of pectin, de-esterification is thought to take place by the action of pectinesterase, which then allows polygalacturonase (polygalacturonide glycanohydrolase, EC 3.2.1.15) to rupture the glycosidic linkage (Jansen & MacDonnell, 1945). The types of polygalacturonases that attack polygalacturonic acid of high molecular weight and low degree of esterification have been divided into two groups (Demain & Phaff, 1957). Exopolygalacturonase removes single galacturonic acid units from the reducing end of polygalacturonic acid, ultimately resulting in complete hydrolysis to galacturonic acid (Patel & Phaff, 1958). Endopolygalacturonase hydrolyses the substrate at random, eventually converting it into a mixture of the monomer and the dimer (Phaff & Demain, 1956). Thus, in spite of conclusions to the contrary (McCready, McComb & Jansen, 1955), there is considerable evidence that tomato fruit contain at least two polygalacturonase components, with much of the activity belonging to the endopolygalacturonase category (Luh, Leonard & Phaff, 1956).

The activity of pectinesterase increases as 'mature' green tomatoes pass through selected colour stages to become fully red (Hobson, 1963). In fruit suffering from a physiological disorder often known as 'blotchy' ripening (Bewley & White, 1926; Cooper, 1957), in which harder regions of differently coloured tissue occur amid the soft red locule walls of ripe specimens, the activity of pectinesterase remained at a level normally associated with fruit in the early stages of uniform ripening. Despite this fact, it was concluded that the enzyme activity in these abnormal areas was not likely to be a critical factor in the disorder. Hence any association between 'blotchy' ripening and the pectic enzyme complex more probably involves alterations in the activity of polygalacturonase. The present paper records the extent of the increase in polygalacturonase activity during the uniform ripening of tomato fruit, and illustrates the changes in the activity of the enzyme that occur in areas of fruit suffering from 'blotchy' ripening.

EXPERIMENTAL

Tomato varieties. Two contrasting varieties, Potentate and Immuna, were used in this study. Brief details of their characteristics, together with the sources of the seed, were given by Hobson (1963).

Tissue samples. The conditions under which the plants were grown and the methods used for obtaining the fruit samples have been reported previously (Hobson, 1963), as have descriptions of the appearance of the fruit at each of the five designated ripening stages (Hobson, 1959). In studies on the distribution of the enzyme activity in tomatoes, a number of fruit were dissected so as to separate the locular contents, the placental tissue and the pericarp. This last was subdivided into outer-locule-wall and inner- or radial-locule-wall tissue. Tissue for the enzyme studies was stored in sealed jars at $-20°$ and analysed within 6 months of sampling. Calibration experiments with normal fruit tissue showed that the activity of polygalacturonase did not alter significantly through storage in these conditions.

Firmness measurements. The firmness of tomato fruit at each of the five ripening stages was assessed with a machine basically designed by Kattan (1957), and subsequently slightly modified (Hobson, 1959). The apparatus measures in arbitrary units the amount of compression resulting from the application of a standard force, the result being known as the 'firmness index' of the fruit. Hence the softer the fruit, the higher the 'firmness index' will be.

Determination of dry matter. Representative samples of about 100 g. of fresh tomato-fruit tissue were dried in a forced-draught oven at 80° for 48 hr.

Enzyme extraction. By using the method of Foda (1957) as a basis, an attempt was made to find more efficient conditions for the desorption of the enzyme from macerated tomato-fruit tissue. Various concentrations and combinations of salts were tested, as listed in Table 1, and as a result the modified procedure given by Hobson (1962) was worked out.

Preparation of various pectic acid substrates. A number of pectic acids were prepared in connexion with experiments on their acceptability as substrates by the enzyme. Wichmann pectic acid and Ehrlich pectic acid C were made by the methods of Newbold & Joslyn (1952*a*, *b*). The procedure outlined by McCready & Seegmiller (1954) was used for the production of McCready pectic acid, and a similar but partially neutralized substrate was prepared according to the recommendations of Kertesz (1951*a*). Enzymic de-esterification of pectin gave Jansen pectic acid (Jansen & MacDonnell, 1945).

Removal of retained solvents from the various preparations was carried out by the method of Kertesz (1951*b*).

Enzyme assay. The method used was extensively modified from that due to Foda (1957). The reaction medium was made by heating together 0·125 g. of Wichmann pectic acid

(Newbold & Joslyn, 1952*a*, *b*), 10 ml. of 0·2 M-potassium hydrogen phthalate, 1 ml. of saturated (at 20°) NaCl solution and about 6 drops of N-NaOH until the solid dissolved. The solution was cooled, passed through a no. 2 sintered glass filter, adjusted to pH 4·25 with further N-NaOH and made up to 47 ml. with water.

A 3 ml. sample of the enzyme solution was added to the substrate held at 27° in a water bath. A 5 ml. sample was removed 7·5 min. later and the reaction stopped by mixing it with 0·05 ml. of Teepol (Shell Chemical Co. Ltd., London) that had been previously filtered and brought to pH 7·35 with 25% (v/v) HCl. Further samples were removed at appropriate time intervals and the enzyme action arrested in a similar manner. The increase in reducing power brought about in the substrate was measured by modifications (Foda, 1957; Hobson, 1962) of the picric acid method devised for reducing sugars by Willaman & Davison (1924).

A unit of activity is defined as the quantity of enzyme contained in 100 g. of fruit tissue that, in the initial stages of hydrolysis at pH 4·25, would produce an increase equivalent to 1·0 mg. of galacturonic acid monohydrate/hr. at 27° from Wichmann pectic acid.

Enzyme activity and pH. In investigating the relation between the activity of the enzyme and the pH of the substrate, the standard amount of potassium hydrogen phthalate buffer was used in each case, and the pH of the substrate adjusted to the various values with N-NaOH. The pH at which the reaction took place was taken as the average of the value for the medium before the reaction started and its value immediately after the final sample had been removed. The drift in pH during the reaction time was usually small.

Polygalacturonase in the expressed sap. Fresh fruit were disintegrated in a macerator and a proportion was placed between a double thickness of Whatman no. 1 filter paper in a 3·125 in. diam. steel cylinder provided with a close-fitting solid steel piston. The sap was expressed from the tissue by using an Apex hydraulic press type A1 (Apex Construction Ltd., London, W. 1) up to 2500 lb./in.^{2}, centrifuged at 2000*g* for 10 min. and finally filtered through a Whatman no. 5 paper with suction. The enzyme in 30 ml. of sap was precipitated by 5 vol. of cold 95% (v/v) ethanol in the usual way. The total enzyme activity contained by the macerate was also determined by the standard extraction procedure.

Polygalacturonase activity in overripe fruit. About forty tomatoes, all at the red stage of ripeness (Hobson, 1959), were chosen from fruit on plants of the variety Immuna. Five or six of these fruit, selected at random, were picked at the beginning of the experiment, and further similar samples were picked at either 3-day or 4-day intervals for up to 18 days. Tissue for determinations of dry matter and for subsequent analysis for polygalacturonase activity was removed in a representative manner from each of the batches of fruit immediately after picking.

The entire experiment was repeated slightly later in the season.

Enzyme inhibitors. Since some polyphenolic substances have been shown to inhibit polygalacturonases from various sources, the polyphenols were extracted from normal and 'blotchy' locule-wall tissue by the method of Hobson (1963). The partially purified extract was made up to 10 ml. with water, and the effect of 4 ml. portions on tomato polygalacturonase activity investigated. In addition, the possible inhibition of polygalacturonase by quantities of phenolic acids demonstrated as being present in 'blotchy' tissue (Walker, 1962) was studied. Solutions containing up to 10 mg. of caffeic acid, chlorogenic acid, ferulic acid [in 4% (v/v) ethanol] and *p*-coumaric acid [in 8% (v/v) ethanol] (L. Light and Co. Ltd., Colnbrook, Bucks.) were added to standard reaction mixtures for measuring the activity of the enzyme.

RESULTS

Extraction and determination of polygalacturonase. Before it was possible to compare the activity of polygalacturonase contained by fruit in various physiological conditions, it was necessary to show that the extraction of the enzyme was efficient, that the conditions for enzyme action were optimum and that the assay method gave reproducible results. Foda (1957), for instance, macerated the tissue for 4 min. with 2·5% of its weight of a 10:1 mixture of sodium chloride and sodium oxalate, but this procedure was shown to bring only a proportion of the enzyme into solution. By extracting the tissue twice more with a 2·5% (w/v) solution of the salt mixture, much of the enzyme remaining in the cellular debris was brought out. Carrying out these manipulations at 3° or below was also shown to lessen the extent to which the enzyme became denatured.

A survey of the relative merits of a number of salt mixtures for the extraction of the enzyme was undertaken, and a summary of the results is given in Table 1. It is seen that the sodium oxalate recommended by Foda (1957), which probably serves to sequester Ca^{2+} ions in the tissue during the extraction, was replaced to advantage by the disodium salt of EDTA. The conclusion from the experiments was that 7·5% of a 10:1 mixture of sodium chloride and EDTA (disodium salt), added first as a solid and then as a solution to the extracted tissue, provided efficient conditions for the desorption of the enzyme.

The success with which polygalacturonase was extracted from red tomato tissue is shown in Table 2. The macerated tissue was treated with the appropriate amount of the solid salt mixture and then by four portions of extractant solution. The enzyme solutions resulting from each of these five extractions were separately precipitated, washed, dissolved and assayed. It was found that, each time the tissue was extracted, about three-quarters of the enzyme in it was removed. Since just over 98% of the total activity brought out in these extractions occurred in the first three stages, limitation to this number of steps was adopted as standard procedure.

Although Foda (1957) recommended the use of potassium hydrogen phthalate as a buffer in the

Table 1. *Efficiency of various salts in the extraction of polygalacturonase from red tomato tissue*

In each experiment, three identical portions (30 g.) of macerated tomato tissue were stirred with the three appropriate mixtures of solid salts. After 15 min., each was centrifuged, the supernatant enzyme extract filtered off and replaced by 20 ml. of a solution of the same mixture of salts at a similar concentration (w/v) as that originally added to the tissue. A further 15 min. later, the enzyme in solution was again removed and the procedure repeated with a second portion of extractant solution, giving in total about 60 ml. of enzyme extract in each case. The procedures for precipitating, dissolving and assaying the enzyme, and also a definition of the units of activity, are given in the text. Different batches of macerated tissue were used for each experiment so that the activities are comparable only within an experiment.

Expt. no.	Concn. of mixture of salts (%, w/w) and solution (%, w/v)	Salt mixture (10:1, w/w)	Units of activity
1	1·25	NaCl–sodium oxalate	400
	2·50	NaCl–sodium oxalate	880
	2·50	NaCl–EDTA (disodium salt)	2000
2	3·00	NaCl–sodium oxalate	1000
	6·00	NaCl–sodium oxalate	1800
	3·00	NaCl–EDTA (disodium salt)	2480
3	2·00	NaCl–EDTA (disodium salt)	1040
	4·00	NaCl–EDTA (disodium salt)	1560
	6·00	NaCl–EDTA (disodium salt)	2080
4	5·00	NaCl–EDTA (disodium salt)	2440
	7·50	NaCl–EDTA (disodium salt)	3000
	10·00	NaCl–EDTA (disodium salt)	2960
5	4·50	NaCl–EDTA (disodium salt)	3040
	6·00	NaCl–EDTA (disodium salt)	3120
	7·50	NaCl–EDTA (disodium salt)	3440

Table 2. *Efficiency of extraction of polygalacturonase from red tomato tissue*

The percentage activity of polygalacturonase in each of five successive extractions of 30 g. of ripe tomato tissue was measured. At each stage the extracted enzyme was precipitated by 100 ml. of cold ethanol, filtered off, dissolved and assayed as detailed in the text. The activities given are the means of two experiments.

Extraction no.	Extractant per 30 g. of tissue	Activity (%)
1	2·25 g. of NaCl–EDTA (disodium salt) (10:1, w/w)	73·4
2	20 ml. of a 7·5% (w/v) solution of the above mixture of salts	18·5
3	20 ml. of the same solution	6·1
4	20 ml. of the same solution	1·5
5	20 ml. of the same solution	0·5

reaction medium for studying pectolytic enzyme activity, he gave no indication of the concentration of the salt he found necessary for stabilization of the pH. In the present investigation, a final concentration of 0·05 M-phthalate in the medium was effective in preventing the pH from drifting more than 0·03 unit from the intended value during an experiment.

Though Wichmann pectic acid is the substrate most commonly used in studies on polygalacturonase, alternative polygalacturonic acids were compared as substrates for the enzyme. Preliminary experiments indicated that a pH optimum of about 4·25 was common to the action of the enzyme on Wichmann pectic acid, Jansen pectic acid or Ehrlich pectic acid. For the McCready pectic acid, however, the activity at this pH was only 70% of that given at an optimum of pH 4·06. Even if this finding is taken into consideration, the results in Table 3 show that the McCready pectic acid was far inferior to the other substrates, between which no significant difference was obvious. Since Wichmann pectic acid was among the most effective of the substrates for polygalacturonase, and was somewhat easier to prepare than the alternatives, its use as a standard substrate in this investigation was continued.

The relation between pH and enzyme activity with both Wichmann pectic acid and Jansen pectic acid as substrate was examined in greater detail. Attempts to fit mathematical equations to the points to calculate the theoretical maxima were not successful, and so the curves were drawn by inspection. The two curves on each Figure represent results from typical enzyme preparations made on different occasions from ripe fruit of the variety Potentate. Wichmann pectic acid (Fig. 1) showed maximum activity in the region of pH 4·23, with a smaller peak about pH 4·49. Jansen pectic acid (Fig. 2) gave maxima at pH 3·81 and 4·31, but the relative magnitude of the peaks differed in one enzyme preparation from the other.

Polygalacturonase activity in uniformly coloured and in 'blotchy' ripened tomato fruit. The procedures developed for the extraction and assay of polygalacturonase were employed to follow the changes in enzyme activity during normal ripening and senescence, and also the alterations in activity that occur in the contrasting areas of 'blotchy' fruit. Because the damage associated with 'blotchy' ripening is largely confined to the outer locule walls of affected fruit, samples of tissue of both whole fruit and outer walls at the various stages of ripeness were analysed for pectolytic enzyme activity. The results for evenly maturing fruit, given in Table 4, show that no part of green fruit nor even the outer locule walls of those at the green–orange stage contained any enzyme activity. Both whole-fruit and outer-wall tissue at the subsequent stages showed activities that rose extremely rapidly as ripening proceeded. By using the mean data previously obtained for the time elapsing between comparable ripening stages (Hobson, 1959), the activity of the enzyme increased exponentially with time from the green–orange to the orange stage, with a further significant increase to the red stage. Polygalacturonase activity in red fruit was almost 200 times as high as that in fruit just beginning to change colour (i.e. at the green–orange stage). At almost all comparable stages of ripeness, Immuna tissue showed greater activity than did Potentate tissue. The differences between the two varieties were particularly marked when considering wall tissue at the red stage; this may be of significance in connexion with the less firm nature of Immuna fruit compared with Potentate (Hobson, 1959). The values in Table 4 make it clear that, immediately after the initiation of enzyme synthesis in locule-wall tissue, the activity rose more rapidly than the average for the whole fruit, resulting in nearly twice as much activity per unit weight of tissue in the red walls than the average activity for the entire fruit.

To find out how far the synthesis of polygalacturonase continued into the period of senescence, a batch of fruit of the variety Immuna were allowed

Table 3. *Relation between the activity of polygalacturonase and various pectic acid substrates*

Each substrate was dissolved to the extent of 0·25% (w/v) in a solution containing standard amounts of potassium hydrogen phthalate and sodium chloride, adjusted to pH 4·25. Details are given in the text, as are those for the preparation of the various substrates. The activities of the substrates are given as proportions of that of the most active, Ehrlich pectic acid C.

Substrate	No. of experiments	Relative activity
McCready pectic acid (unneutralized)	2	17·7
McCready pectic acid (neutralized)	2	35·2
Wichmann pectic acid	9	95·1
Jansen pectic acid	5	98·8
Ehrlich pectic acid C	5	100·0

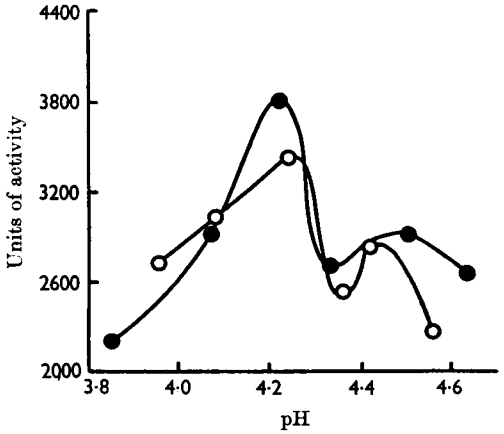

Fig. 1. Effect of pH on the activity of polygalacturonase with Wichmann pectic acid as substrate. The reaction medium, kept at 27°, consisted of 0·125 g. of pectic acid dissolved in a solution of potassium hydrogen phthalate (final concn. 0·04 M) and 1 ml. of saturated NaCl, and adjusted to the appropriate pH value with N-NaOH (final vol. 50 ml. after the addition of enzyme). An initial sample was taken 7·5 min. after the addition of 3 ml. of the enzyme solution and a final sample 20 min. later. The samples were analysed for increased reducing power as detailed in the text. ○ and ● represent results from extractions of the enzyme from separate batches of red tomato fruit of variety Potentate.

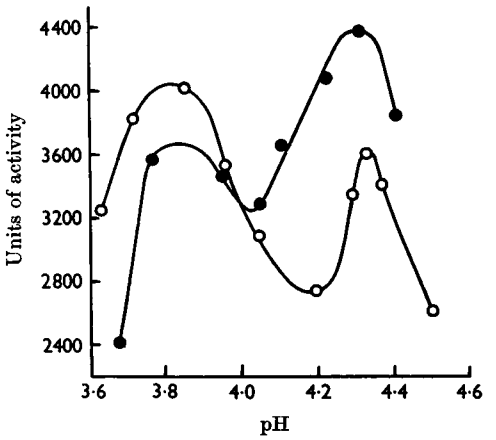

Fig. 2. Effect of pH on the activity of polygalacturonase with Jansen pectic acid as substrate. The experimental details were as given for Fig. 1. ○ and ● are the results from extractions of the enzyme from separate batches of red tomato fruit of variety Potentate.

Table 4. *Polygalacturonase activity in uniformly ripening tomato fruit*

The units of activity are measured as increased reducing power, in terms of mg. of α-D-galacturonic acid monohydrate/hr./100 g. of fresh tissue. Mean values have been obtained by averaging the logarithms of individual experimental values; the logarithms were also used for statistical analysis so that the error variances of the values for the various stages of ripeness were kept approximately the same. The means in logarithmic form are given in parentheses. The assay method was as given in the text.

Stage of ripeness	Green	Green–orange	Orange–green	Orange	Red	No. of experiments	Least significant difference between means ($P = 0.05$)
Potentate whole fruit	0	15	187	912	3630	5	0·28
	(—)	(1·19)	(2·27)	(2·96)	(3·56)		
Dry matter (%)	—	6·15	6·34	6·17	6·08		
Immuna whole fruit	0	19	251	1150	3980	5	0·16
	(—)	(1·27)	(2·40)	(3·06)	(3·60)		
Dry matter (%)	—	6·31	6·45	6·16	6·46		
Potentate outer locule walls	0	0	204	1780	6920	3	0·28
	(—)	(—)	(2·31)	(3·25)	(3·84)		
Dry matter (%)	—	—	7·46	7·25	7·33		
Immuna outer locule walls	0	0	240	1550	7590	3	0·17
	(—)	(—)	(2·38)	(3·19)	(3·88)		
Dry matter (%)	—	—	6·74	6·62	6·78		

Table 5. *Polygalacturonase activity in overripe tomato fruit (variety Immuna)*

Unharvested fruit at the red stage of ripeness were selected and five or six of their number (chosen at random) were picked at intervals during the next 18 days. Samples of these fruit were then analysed for polygalacturonase activity. The units of activity and assay methods are as given in the text. The activity and dry-matter values are the means of two experiments.

Time after the red stage of ripeness (days)	Units of activity	Dry matter (%)
0	4100	6·26
3	4300	6·65
6	4680	6·48
10	5910	6·76
14	5790	6·46
18	4690	6·64

to remain on the plants after they had reached the red stage of ripeness, and samples of their number were taken for analysis every few days. As the values in Table 5 indicate, the activity rose until at least 10 days after the red stage of ripening. The fall in activity recorded thereafter may well have been merely due to an increasing number of the fruit (probably containing high activity of the enzyme) becoming diseased, leaving those that held rather lower activities to be sampled.

A comparison of polygalacturonase activity in the two differently coloured regions of 'blotchy' fruit (Table 6) reveals that for whole-fruit tissue the green areas had only 17 and 15 % of the activity in the red areas, and for outer-locule-wall samples 5 and 11 % of that of the red parts, for the varieties Potentate and Immuna respectively. The differences were highly significant in all cases. Tissue from the green areas also had a lower average content of dry matter than that from the red areas, but the differences were not statistically significant.

Under commercial growing conditions, 'blotchy' ripened fruit tend to be left on the plant rather longer than normal, and hence the red part of the fruit is probably in a more mature condition than if the whole had been uniformly coloured. Thus the results in Table 6 might be expected to show that the red tissue from non-uniformly coloured fruit contained more polygalacturonase activity than was normally found in red tissue from evenly coloured fruit. But in none of the cases in Table 6 did the red areas of 'blotchy' fruit give an average of more than two-thirds the activity found in tissue from evenly red fruit (Table 4). Hence the red parts of 'blotchy' fruit cannot be regarded as typical of this colour stage, and the fruit as a whole is exceptional (see Winsor & Massey, 1959; Hobson, 1963).

Despite a number of reports of the inhibition of polygalacturonase by phenolic and other substances (Cole, 1958; Pollard, Kieser & Sissons, 1958; Porter & Schwartz, 1962), none of these has referred to the tomato fruit. The phenolic compounds extracted from both uniformly red and 'blotchy' fruit tissue did not inhibit tomato polygalacturonase activity. The addition *in vitro* of amounts of caffeic acid, chlorogenic acid, ferulic acid and *p*-coumaric acid, up to a concentration of 0·02 %, was also without effect on the enzyme

Table 6. *Polygalacturonase activity in red and green regions of tomato fruit showing 'blotchy' ripening*

The units of activity and assay methods are as given in the text. Each activity value is the mean of four experiments, calculated after conversion into logarithms to compensate for the differing error variances. The means in logarithmic form are given in parentheses.

	Units of activity		Activity in green areas (as percentage of the red)	Least significant difference between means ($P = 0.05$)
	Red areas	Green areas		
Potentate whole fruit	2240 (3·35)	380 (2·58)	17	0·15
Dry matter (%)	5·62	5·60		
Immuna whole fruit	1380 (3·14)	209 (2·32)	15	0·16
Dry matter (%)	6·39	6·27		
Potentate outer locule walls	3550 (3·55)	166 (2·22)	5	0·51
Dry matter (%)	5·37	5·19		
Immuna outer locule walls	2340 (3·37)	269 (2·42)	11	0·30
Dry matter (%)	5·76	5·39		

activity. This evidence, together with the reported absence of excessive amounts of polyphenolic acids in 'blotchy' tissue (Walker, 1962), make it unlikely that phenolic inhibitors are involved in the disorder.

Polygalacturonase activity and the firmness of tomato fruit. The 'firmness index' of fruit of varieties Potentate and Immuna at five stages of ripeness has been established by Hobson (1959). Polygalacturonase activities in the same varieties and at similar ripening stages are given in Table 4. Though it must be stressed that the data for firmness and for enzyme activities were obtained in different seasons, the standard error for each set of results is such that general inferences about fruit behaviour are valid. The relation between firmness and polygalacturonase activity is shown in Fig. 3, and the experimental points are seen to fit closely to calculated curves containing terms of ascending degree to the fifth power of the 'firmness index'.

Distribution of polygalacturonase in ripening tomato fruit. If the inception of ripening is taken as the initial appearance of yellow colour on the fruit, the region in which this colour change is first detectable is not uniform even within one variety of tomato. Typically, the first yellowness is seen in the placenta of the fruit or in the semi-liquid matrix immediately surrounding the seeds. Occasionally, however, the pericarp tissue, especially towards the blossom end of the fruit, turns yellow first, rapidly followed by a change in colour of the placenta and the outer locule walls. If it could be shown that polygalacturonase activity in the fruit closely followed the normal sequence of coloration then this would constitute additional evidence that the enzyme was intimately connected with the ripening mechanism.

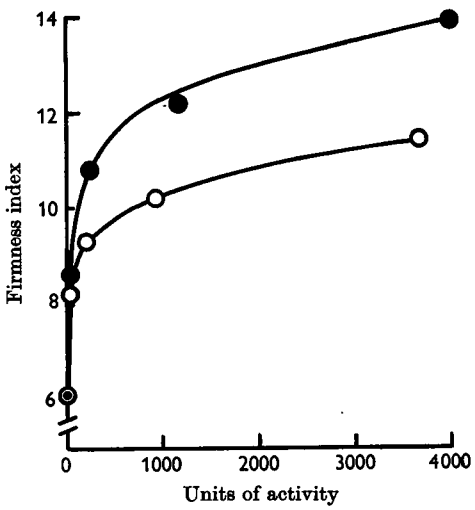

Fig. 3. Relation between the activity of polygalacturonase and the firmness of tomato fruit, represented by the 'firmness index', at five defined stages of ripeness. The enzyme activities (Table 4) and the firmness data (Hobson, 1959) are based on different batches of fruit. Curves of the fifth order are shown which were calculated to fit the experimental points, represented by ● for fruit of the variety Immuna and ○ for variety Potentate.

Immuna fruit just more mature than the green–orange stage (so that the outer locule walls showed at least some activity) were dissected and then the enzyme activity was determined in each of the distinct parts. From the results in Table 7, where polygalacturonase activities are reported/100 g. of tissue, the outer walls are seen to contain the highest activity, followed in turn by the inner

Table 7. *Distribution of polygalacturonase in ripening tomato fruit (variety Immuna)*

Tomato fruit slightly more mature than the green–orange stage were dissected and the polygalacturonase activity in the various divisions was determined. The units of activity and assay methods are as given in the text. The number of experiments upon which each column of values is based is given in parentheses.

	Tissue in each division (%)	Units of polygalacturonase activity	Activity in each division (% of total)
Outer locule walls	54·0 (3)	20·1 (5)	46·9 (5)
Inner locule walls	10·0	13·6	31·6
Placenta	6·4	9·2	21·5
Locular contents	29·6	0·0	0·0

locule walls and the placental tissue. No activity was detected in the locular contents. Batches of progressively less mature tomatoes were examined similarly. In almost all cases it was found that activity persisted longest in the placental tissue. Hence, as the fruit began to ripen, polygalacturonase activity developed first in that region which most often showed incipient yellowness, the placenta. Soon afterwards activity was found in both the inner and outer locule walls. These facts lend considerable weight to the suggestion that the increases in enzyme activity and the process of maturation are closely integrated.

To establish whether the increasing activity of polygalacturonase during ripening was associated more with the walls of the cells or with the cell sap, samples of fresh tomatoes at five stages of ripeness were macerated and the total activity was compared with that of the juices expressed from the same tissues. Assuming that comparable amounts of juice were expressed from tissue at each of the stages of ripeness, the percentage of the total activity that can be attributed to the juices decreased progressively with maturity, from 21·6 at the green–orange stage to 3·7 at the red stage. The actual activity shown by them rose because of the intense synthesis of the enzyme during ripening (Table 4). The values are consistent with the idea that the enzyme is synthesized in the central vacuole of the cell and that increasingly strong adsorption of the enzyme on to the cell walls takes place during ripening.

DISCUSSION

Extraction and assay of polygalacturonase. The methods published previously for the extraction of polygalacturonase (Kertesz, 1938; McColloch & Kertesz, 1949; McColloch, Keller & Beavens, 1952; McCready *et al.* 1955; Luh *et al.* 1956; Patel & Phaff, 1960*a*) used various proportions of sodium chloride mixed with the pressed pulp of the fruit (since the juice contained relatively little of the enzyme), and Foda (1957) recommended that a proportion of sodium oxalate be mixed in as well. None of these methods proved to be quantitatively satisfactory and further development was necessary before this was achieved.

Wichmann pectic acid is produced by the alkaline de-esterification of pectin. Both Newbold & Joslyn (1952*a*, *b*) and Luh & Phaff (1954) indicated that the molecular weight of this acid was about 25000. McCready pectic acid, on the other hand, results from acid hydrolysis of the side chains of pectin, and is also acid-soluble. A short chain length is therefore to be expected and Patel & Phaff (1959) reported a molecular weight for it of 1800–2000. A combination of both acidic and alkaline hydrolysis was used during the preparation of Ehrlich pectic acid C, and its molecular weight is said to be about 14000 (Newbold & Joslyn, 1952*a*, *b*). Jansen pectic acid was made by enzymic demethoxylation and may well have a molecular weight approaching that of the original pectin, perhaps as high as 62000 (Newbold & Joslyn, 1952*a*, *b*). The action of tomato polygalacturonase did not appear to depend primarily on the molecular weight of the substrate, and all the pectic acids prepared except McCready pectic acid gave very similar rates of reaction.

A number of investigators (McColloch & Kertesz, 1948; Foda, 1957; Patel & Phaff, 1960*b*) have concluded that tomato fruit contain at least two polygalacturonase components. This mixture of pectolytic enzymes probably accounts for the wide variations in the optimum pH that have been quoted. Maxima for the activity of tomato polygalacturonase have included pH 2·5 (Patel & Phaff, 1958, 1960*b*) with short-chain pectic acid, and pH 3·5 (Foda, 1957), pH 4·0 (MacDonnell, Jansen & Lineweaver, 1945), pH 4·5 (McColloch & Kertesz, 1948; Roelofsen, 1953; Luh *et al.* 1956; Patel & Phaff, 1958) and pH 5·0 (Foda, 1957; Patel & Phaff, 1960*b*) with normal pectic acid.

The relation between pH and pectolytic activity during ripening was interpreted by Foda (1957) as indicating the presence of both polygalacturonase and 'depolymerase' (McColloch & Kertesz, 1948). The latter enzyme was thought to make a decreasing contribution towards full ripeness of the tomato, but only incomplete evidence was given in support of the possibility of two distinct components.

Although the pectic content undoubtedly decreases during the ripening of tomato fruit (LeCrone & Haber, 1953; Hobson, 1963), the mechanisms *in vivo* do not appear to result in the complete degradation of the molecule to the monomer, since no comparable increase in galacturonic acid occurs.

The ability of tomato pectolytic enzymes to hydrolyse pectic acid completely to galacturonic acid is not in doubt (Luh *et al.* 1956), although the final stage in the degradation is a slow one. The rapid lowering of the viscosity compared with the small increase in reducing power of pectic acid was thought to indicate a preponderance of endopolygalacturonase, although the immediate release of some galacturonic acid by pectolytic enzyme preparations probably represents the action of exopolygalacturonase. The situation was further complicated by the suggestion of Patel & Phaff (1958) that the endopolygalacturonase contained two components with different pH optima. The conclusion of Demain & Phaff (1957) was that the balance between exopolygalacturonase and endopolygalacturonase in tomatoes varied according to the conditions of enzyme preparation. This might explain the differences in activity found at the two maxima between the two preparations used in obtaining the pH–activity relations for Fig. 2. It is, however, increasingly apparent that pectolytic enzyme extract from tomato fruit must be regarded as a mixture of enzymes, each capable of degrading a limited and characteristic number of substrates at reaction rates dependent on the pH of the cell and the precise chain length involved (McColloch, 1948; Patel & Phaff, 1960*b*). Some of the observations in the present study are most easily explained in terms of such a mixture, but confirmation must await a more detailed physical separation of the polygalacturonases in tomato fruit.

Polygalacturonase in normal and abnormal ripening. During the maturation of tomatoes, the 'climacteric rise' in respiration probably coincides with the first appearance of orange colour on the fruit (Clendenning, 1942; Workman, Pratt & Morris, 1957), and from this point polygalacturonase activity becomes increasingly evident. Loss of firmness by the fruit also occurs as ripening proceeds (Kattan, 1957; Hobson, 1959). It is of interest to consider the possibility of a relation between polygalacturonase activity and the firmness of different varieties of tomato at comparable stages of ripeness. Foda (1957) noted that the firmer of two varieties of tomato he was investigating also exhibited the lower pectolytic activity. Further, red fruit of the variety Potentate were both firmer (Hobson, 1959) and lower in polygalacturonase activity (Table 4) than those of variety Immuna. In the light of these findings, the distinct relation between the firmness of these last two varieties of fruit and the polygalacturonase activity strongly indicates that, for these varieties at least, the two important ripening characteristics of loss of firmness and increased polygalacturonase activity are positively associated. Further investigations into the relation between these two factors might not only provide direct evidence for the involvement of the pectic enzymes in ripening but might also be of use in selecting firm lines in a breeding programme.

In contrast with the 200-fold rise in activity from green–orange to red fruit found in the present study, the results of Foda (1957), with American tomatoes, indicated only a sevenfold increase from the 'turning stage' to the maximum of (presumably) ripe fruit. No complete definition of the units in which the activity was measured was provided, so that direct comparison with the values given in Table 4 is not possible. Foda (1957) nevertheless clearly showed that the enzyme activity in American varieties of fruit reached a maximum at about 12 days after the green–orange stage (when the fruit were in all probability well past the red stage of ripeness), followed by a decrease in activity; this parallels the trend in results from the present work (Table 5).

It was concluded by McColloch *et al.* (1952) that the pectic enzymes were localized near the surface of ripe fruit and that a deeper red colour was associated with the higher activities of the pectic enzymes. Although their work was not concerned with ripening disorders as such, their conclusions on the localization of the pectic enzymes in fruit and the close relation between enzyme activity and the colour of the fruit flesh are fully in accordance with those from the present investigation.

The rise in polygalacturonase activity during ripening may well be due to a synthesis of protein. In the apparent absence of effective quantities of phenolic inhibitors in abnormal tomato tissue, it is tempting to conclude that the serious deficit of polygalacturonase in 'blotchy' ripened tissue is due to a breakdown in the mechanism that leads to its synthesis. This in turn prevents the characteristic changes associated with ripening from taking place in affected areas, leaving them green and considerably harder than normal. However, without more detailed knowledge of the mechanism of fruit ripening, it is difficult to deduce whether the failure in enzyme synthesis is a cause or an effect of the disorder. Nevertheless, no evidence has come to light during the present investigation that diminishes the implication of the pectic enzymes in fruit ripening. It is, in fact, becoming more evident that the physical characteristics of fruit tissues are a reflexion of the activities of pectic enzymes to which they are subjected.

SUMMARY

1. An efficient extraction system for the desorption of polygalacturonase from fruit tissue has been developed.

2. Five pectic acid preparations have been com-

pared as substrates for polygalacturonase and the pH–activity relations investigated.

3. Polygalacturonase activity was determined during the ripening of fruit of two varieties of tomato. No polygalacturonase activity was found in green fruit; thereafter the activity rose exponentially with time to the orange stage, increasing further to the red stage.

4. Enzyme activity continued to rise as fruit became overripe.

5. The well known physiological disorder, 'blotchy' ripening, led to a lowered activity in the apparently normal red area, and to a severe decrease in activity in the abnormal non-red area.

6. The distribution of polygalacturonase in parts of the fruit during ripening has been studied.

7. It is concluded that 'blotchy' ripening is associated with a failure in the synthesis of polygalacturonase, although it is uncertain whether this lack of the enzyme is a cause or an effect of the disorder.

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