A Study of Enzymes that can Break Down Tobacco-Leaf Components

3. FUNGAL POLYGALACTURONASE ON LEAF FIBRE

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(Received 20 January 1950)

The breakdown of pectic substances in plant material by pectic enzymes is of importance in many connexions. Among these are the changes which occur in the ripening of fruit, and in fruit and vegetables during storage; in the process of retting; in the decomposition of plant material in the soil; and in the liberation of viruses from plant tissue. It is this last aspect which was the main reason for undertaking the present investigation on the effect of pectic enzymes on leaf tissue. The action of pectin esterase, which converts pectin to pectic acid, in ground leaf material has already been studied (Holden, 1945). A number of workers (Harter & Weimer, 1921; Menon, 1934; Holden, 1950) have investigated the effect on plant tissue of extracts from fungi, but the extracts certainly contained other enzymes besides those concerned with the breakdown of pectic substances. The effects on leaf tissue of purified polygalacturonase, free from pectin esterase, amylase and cellulase activity, have now been studied. The enzyme, which was kindly given by Dr H. Line-weaver, was made from Pectinol 45 AP (Rohm and Haas Co.) and is referred to here as PG. The method of preparation was described by Lineweaver, Jang & Jansen (1949).

MATERIAL AND METHODS

The methods described in the first paper of this series (Holden, Pirie & Tracey, 1950) were used for preparing tobacco-leaf fibre and for determining carbohydrates.

Uronic acid was determined by the manometric method (Tracey, 1948).

Calcium was determined on ashed fibre and in extracts from fibre by precipitation as calcium oxalate, the precipitate separated by centrifugation, dissolved in H_2SO_4 and titrated with 0.01 N-KMnO₄.

Ammonia. Samples made alkaline by addition of sodium borate were steam distilled in a Markham (1942) still. The liberated NH_3 was absorbed in boric acid and titrated with HCl.

Oxalic acid. Fibre (10 g. wet wt.) was extracted three times with 20 ml. lots of 2N-HCl. The combined extracts were centrifuged and to a portion of the supernatant solution, $CaCl_2$ solution was added and the mixture was then neutralized with 2N-ammonia solution. The precipitated calcium oxalate was centrifuged down, after standing overnight, dissolved in H_2SO_4 and titrated in the hot with 0.01 N-KMnO₄. When small amounts of oxalic acid in large

volumes of solution were determined, a correction, to allow for the slight solubility of calcium oxalate, was applied, based on the values given by known amounts of oxalic acid precipitated in the same volume and under the same conditions.

EXPERIMENTS AND RESULTS

Incubation of fibre with enzyme

Jansen & MacDonnell (1945) showed that removal of methoxyl groups from pectin is necessary before PG can hydrolyse the 1:4- α -glycosidic links of the polygalacturonide chain. Fibre was therefore neutralized by suspending it in water and making small additions of NaOH at intervals, or by soaking in neutral phosphate solution to bring about pectin esterase action (Holden, 1945). Although sodium pectate is soluble in water, very little of the pectic acid in leaf fibre becomes soluble on neutralization at room temperature. Heating in dilute alkaline solution causes some pectic material to become soluble.

Most experiments were done on fibre from which starch had been removed by incubation with saliva or a commercial trypsin preparation (Holden & Tracey, 1950). Fibre was incubated with PG in sodium acetate buffer solution (pH 4.5) at about 35° in the presence of $CHCl_3$. After several incubations with fresh lots of PG (0.1 mg./g. wet wt. of fibre), the fibre was soft and partly broken up. The cells separated from each other when the lumps of tissue were pressed out with a glass rod; the middle lamella was attacked but the cellulose wall left intact. Fibre incubated with PG resembles fibre extracted with hot ammonium oxalate solution. Polyuronide (PU) determinations on the residue after incubation with PG showed, however, that over 40% of the original PU remained after three or four incubations, while extraction with ammonium oxalate removed about 90% of the PU. The effect of various pretreatments of the fibre on the subsequent action of PG was therefore investigated. Pretreatments which had no appreciable effect either on the rate of release or amount of carbohydrate released were extraction of the fibre with fat solvents, boiling a suspension of the fibre and freezing the fibre. Pretreatments which had a marked effect were fine grinding of the fibre in a triple-roller mill (Bawden & Pirie, 1944) and extraction with dilute acid or other agents which remove Ca from the fibre.

Effect of milling

When fibre was finely ground in the triple-roller mill, the rate of liberation of carbohydrate on subsequent incubation with PG was increased and the total amount released in three incubations was slightly more from milled than

minced fibre (Table 1). If starch was not removed before milling, the extracts from incubations with PG contained starch, or its breakdown products, and the carbohydrate content of the fluid was no longer a reliable measure of PG action.

Table 1. *Effect of milling on action of polygalacturonase on tobacco-leaf fibre*

(1 g. lots of trypsin-treated fibre (dry matter 17.0%) + 2 ml. 0.2M-sodium acetate buffer (pH 4.5) + 1 ml. PG solution (0.1 mg./ml.) + 5 ml. water for each incubation of 24 hr. at 38°.)

	mg. carbohydrate liberated/100 mg. dry fibre	
	Minced	Milled
First incubation	11.6	16.5
Second incubation	6.1	5.2
Third incubation	3.2	1.6
Totals	20.9	23.3

Decalcification of fibre and its effect on PG action

Calcium ions strongly inhibit the action of PG on extracted pectic acid; methods of removing Ca from fibre were therefore investigated to determine the effect on subsequent PG action. All the Ca can be removed with HCl, but such a low pH is needed to achieve this that there is risk of other changes being brought about. In the presence of other cations about 70% of the Ca can be removed at higher pH's. This exchange can easily be effected by percolating through a column of fibre a solution of 0.5M ammonium, sodium, or potassium acetate adjusted to pH 4-5 or a mixture of NH_4Cl and acetate buffer at pH values between 3.5 and 5. Acetate or chloride solutions, used at pH 6-7, will remove about 15% of the Ca, or only about one-fifth of that extracted at pH 3.5-5. There is little difference from a practical point of view between NH_4 , Na, and K salts for removing Ca; NH_4 was used for most experiments.

In one experiment, 700 ml. of a solution (0.67M- NH_4Cl in 0.033M-sodium acetate buffer, pH 3.5) was percolated through 10 g. starch-free fibre (2 g. dry matter); this reduced the Ca content (in terms of the dry matter) from 3.67 to 0.61%. The Ca content was then further reduced to 0.04% by subsequent extraction with 0.05N-HCl.

The state of calcium in fibre. Calcium oxalate is known to be present in tobacco leaves (Ridgway, 1916). To obtain a maximum figure for the amount of Ca that might be combined with pectic substances in fibre, total Ca and oxalic acid were determined. One batch of fibre, untreated except for washing, had a Ca content of 2.20%, and an oxalic acid

content of 1.65% of the dry matter. This amount of oxalate is equivalent to 35% of the Ca. 8 g. lots of this fibre were extracted with 800 ml. of NH_4Cl -acetate buffer solution (pH 3.5) and 800 ml. water respectively. The results are given in Table 2. This shows that much of the calcium oxalate, in addition to Ca combined with pectic substances, is removed by the NH_4Cl -acetate treatment. Experiments with known amounts of calcium oxalate and ammonium acetate solutions showed that calcium oxalate was soluble to the extent of about 8 mg./100 ml.

Exchange of cations in fibre. The uptake of NH_4^+ ions by the fibre when treated with ammonium acetate solution was compared with the loss of Ca^{++} . When untreated or trypsin-treated fibre was used, the Ca removed was more than equivalent to the NH_4 taken up. This discrepancy is due to removal of some of the calcium oxalate in the ammonium acetate extract. To overcome interference by oxalate, fibre was first treated with acid so that oxalate was removed, and then allowed to absorb Ca from a solution of calcium acetate. When the fibre was treated with 0.5M-ammonium acetate (pH 5), the NH_4 taken up was almost exactly equivalent to the Ca removed.

Fig. 1. Uptake of Ca by decalcified tobacco-leaf fibre. 1 g. lots of acid-extracted fibre (25.5% dry matter) were soaked in calcium acetate solutions of known Ca content for 30 min. Ca was determined in the extracts.

The amount of Ca taken up by fibre from solutions of Ca salts was investigated, and the results of one experiment are shown in Fig. 1. 1 g. lots of acid-extracted fibre were soaked in calcium acetate solutions for 30 min., the extracts filtered off and Ca determined in them. Several concentra-

Table 2. *Extraction of calcium and oxalic acid from tobacco-leaf fibre*

(Separate 8 g. lots of fibre (dry matter 25.7%, Ca 2.20% of dry matter) were treated. Lot 1: with 60 ml. 2N-HCl. Lot 2: successively with eight lots of 100 ml. water, then with 60 ml. 2N-HCl. Lot 3: successively with eight lots of 100 ml. NH_4Cl -acetate solution and then with 60 ml. 2N-HCl.)

	Lot 1		Lot 2		Lot 3	
	Calcium (mg.)	Oxalic acid (mg.)	Calcium (mg.)	Oxalic acid (mg.)	Calcium (mg.)	Oxalic acid (mg.)
In 800 ml. combined water extracts	—	—	7	<7	—	—
In 800 ml. combined NH_4Cl -acetate extracts	—	—	—	—	37.1	24
In 60 ml. 2N-HCl extract	42.4	33.9	35.2	23.4	6.4	7.8

tions and different volumes of the same concentration were used. The pH of the calcium acetate solution fell from approx. 7 to 4.1-4.6 when in contact with the fibre. The fibre took up Ca rapidly from solution and as much was taken up in a few minutes as when left to soak overnight. Until about half saturation of the fibre with Ca was reached, the fibre took up the greater part of the Ca present, even from dilute solution. As the fibre became more nearly saturated, it picked up an increasingly smaller proportion of the total Ca in solution.

Removal of polyuronide from fibre. Extraction of starch-free fibre with salt solutions and dilute acid removed a negligible amount of carbohydrate. After extraction with acid water can easily be squeezed from the fibre, but when it is suspended in water and dilute NaOH is added to bring the suspension to pH 6-7 the fibre swells and becomes very soft. When squeezed out in a cloth it is then difficult to remove the water, and as much as half of the polyuronide material of the fibre is removed with one extraction. Pectic acid can be precipitated from the extracts by addition of acidified ethanol and separated by filtering through madapolam with squeezing.

Decalcification of fibre has a profound effect on the liberation of carbohydrate by PG; the rate of liberation is greatly increased and so is the total amount of carbohydrate set free. Table 3 compares the rates of liberation of carbohydrate, during a single incubation with PG, from untreated and decalcified fibre. Table 4 shows the carbohydrate liberated in successive incubations with PG. For the experiment given in Table 4, the amount of enzyme used for the untreated fibre was ten times that used for the decalcified fibres. However, carbohydrate was released slowly from the untreated fibre and the amount was small, particularly when compared with that from the decalcified fibre with comparable PU content. Over 90% of the PU was removed from the decalcified fibres, the residues having a PU content so low as to make the exact measurement uncertain. Only 11% of the total PU was removed from the untreated fibre. The removal of PU accounts for about 60% of the carbohydrate in the extracts from the decalcified fibre. The reducing sugar (Hagedorn and Jensen method as modified by Hanes (1929), expressed as galacturonic acid) in the extracts is between 30 and 50% of the total carbo-

hydrate; the higher figure is for the more completely decalcified fibre. The amount of carbohydrate in the control for the fibre with the lowest Ca content is about one-quarter of that removed with enzyme, but the reducing sugar is a smaller percentage of the total carbohydrate, indicating that only partially degraded pectic acid is being leached out.

Table 3. *Effect of decalcification on rate of liberation of carbohydrate from tobacco-leaf fibre by polygalacturonase*

(Portions of fibre containing 42-50 mg. dry matter + 1 ml. 0.2M-sodium acetate buffer (pH 4.6) + 2.8 ml. water + 0.2 ml. PG solution (0.1 mg./ml.). Incubated at 37° for times stated. Controls without added enzyme.)

Time of incubation (hr.)	mg. carbohydrate liberated/100 mg. dry fibre		
	Untreated fibre	Decalcified fibre	
	(PU 23.6%, Ca 3.76% of dry matter)	(PU 25.8%, Ca 0.67% of dry matter)	(PU 26.8%, Ca 0.04% of dry matter)
1	1.9	25.7	45.7
4	2.6	37.5	—
16	5.7	37.5	45.7
Control, 16	0.8	1.4	19.4

Extracts obtained by incubating partly decalcified fibre with PG give a gelatinous precipitate on addition of ethanol. The precipitate is less fibrous than pectic acid precipitated under similar conditions. The material, which accounts for about half of the total carbohydrate in the extract, has a uronic acid content of approx. 60%, and a Ca content of 1-2% of the dry matter. When the material is redissolved in water it has a low reducing value, and is only very slowly attacked by PG. However, if acidified ethanol is used for precipitation or the solution is acidified and dialysed before precipitation, the material can then be attacked by PG. The acid treatments lead to removal of Ca from the material, which appears to be partly degraded pectic acid combined with some Ca and possibly with other carbohydrates. The

Table 4. *Effect of decalcification on amount of carbohydrate liberated by polygalacturonase from tobacco-leaf fibre*

(Portions of fibre containing 220-240 mg. dry matter + 2 ml. 0.2M-sodium acetate buffer (pH 4.5) + water and PG solution to give 8 ml. For decalcified fibres 0.005 mg. PG, and 0.05 mg. for untreated. Successive incubations for 24 hr. at 38° with fresh lots of enzyme. Controls without added enzyme.)

	mg. carbohydrate liberated/100 mg. dry matter								
	Untreated fibre			Decalcified fibre					
	(PU 20.2%, Ca 2.70% dry matter)			(PU 21.4%, Ca 0.69% dry matter)			(PU 12.9%, Ca 0.2% dry matter)		
	Total	Reducing	Uronide	Total	Reducing	Uronide	Total	Reducing	Uronide
First incubation	2.7	1.6	—	23.4	6.7	—	17.5	7.8	—
Second incubation	2.7	1.3	—	11.6	3.2	—	2.0	0.8	—
Third incubation	3.6	1.6	—	2.1	0.8	—	1.0	0.5	—
Fourth incubation	2.7	1.6	—	—	—	—	—	—	—
Totals	11.7	6.1	2.2	30.2	10.7	19.9	20.5	9.1	11.5
Control, three incubations	Approx. 1.5	—	—	2.8	Approx. 0.3	—	5.3	Approx. 0.5	—

addition of ethanol to extracts, obtained on incubating almost completely decalcified fibre with PG, gives a small amount of precipitate which is not gelatinous. The precipitate represents about 30% of the total carbohydrate in the extract and has a PU content of about 50% of the dry matter.

Conditions of incubation affecting PG action

The amount of purified enzyme which was available was limited; most experiments were therefore done on decalcified fibre, since more digestion could then be achieved with smaller amounts of enzyme. Experiments were done at pH 4.5, which is the optimum for carbohydrate liberation from the fibre by the enzyme. Fig. 2 shows the effect of using different amounts of PG on the same weight of decalcified fibre. Clearly a satisfactory effect is obtained by using 0.1 mg. PG/g. dry weight of fibre.

Effect of salts. Table 5 shows that high concentrations of NaCl diminish the liberation of carbohydrate from fibre by PG, but that lower concentrations increase it. With 17 hr. incubation the results are qualitatively the same as with only 1 hr. incubation. The greatest losses of uronic acid from the fibre occur at salt concentrations where there is greatest loss of total carbohydrate.

Fig. 2. Variation in activity with amount of polygalacturonase acting on tobacco-leaf fibre. 250 mg. lots of fibre (dry matter 24.8%, Ca 0.69% of dry matter, PU 21.4% of dry matter) + 1 ml. 0.2M-acetate buffer (pH 4.6) + water and PG solution to give total volume 4 ml. Incubated 1 hr. at 36°.

Table 5. *Effect of sodium chloride on liberation of carbohydrate from tobacco-leaf fibre by polygalacturonase*

(400 mg. lots of trypsin-treated decalcified fibre (dry matter 17.8%, Ca 0.64% of dry matter, PU 18.0% of dry matter) + 2 ml. NaCl solution + 1 ml. water + 1 ml. PG solution (0.005 mg./ml.). Incubated for 1 and 17 hr. at 38°. Residues after 17 hr. incubation weighed and PU determined. Water substituted for enzyme solution in control.)

Final concentration of added NaCl (M)	mg. carbohydrate liberated/100 mg. dry fibre		PU content as percentage dry residue (17 hr. incubation)	PU loss as percentage of original dry fibre
	1 hr.	17 hr.		
1.0	0.56	0.79	—	—
0.5	2.47	4.95	15.88	2.65
0.25	5.40	12.15	10.96	7.69
0.1	7.08	13.38	9.40	11.25
0.05	8.10	13.93	10.20	10.33
0.025	6.40	11.69	13.32	7.80
0.01	4.83	10.10	14.20	4.76
No salt	2.70	5.05	16.88	1.53
Control, no salt	0.56	0.79	17.92	0

Table 6. *Effect of calcium on liberation of carbohydrate from tobacco-leaf fibre by polygalacturonase*

(500 mg. lots of trypsin-treated, decalcified fibre (dry matter 21.8%; PU, 25.1% of dry matter; Ca, 0.07% of dry matter) + 1 ml. 0.2M-sodium acetate buffer (pH 4.5) + 2 ml. CaCl₂ solution + 1 ml. PG solution (0.005 mg./ml.). Incubated for 1 and 16 hr. at 37°. Residues after 16 hr. incubation washed with water, collected, weighed and PU determined. Water substituted for enzyme solution in controls.)

Final concentration of added CaCl ₂ (M)	mg. carbohydrate liberated/100 mg. dry fibre		PU removed as percentage of dry fibre	PU content as percentage of dry residue	PU removed as percentage of PU originally present
	1 hr.	16 hr.			
0.025	0.88	1.32	1.1	23.9	4.4
0.01	1.04	2.28	2.9	23.2	11.7
0.005	4.32	6.61	6.0	22.4	23.8
0.002	9.44	17.90	12.5	16.4	50.2
0.001	12.00	24.00	17.3	12.1	69.0
0.0005	20.80	27.00	21.8	5.4	87.3
0.00025	23.50	28.80	22.2	4.8	88.6
No Ca	20.50	28.70	21.1	6.4	84.3
Control, no Ca	2.88	15.96	11.2	18.8	44.7
Control, 0.005	1.20	2.28	1.9	24.3	7.7

As the removal of Ca from fibre had such a marked effect on PG action, it is not unexpected that adding it back to decalcified fibre has an inhibitory effect. Table 6 gives the results of an experiment in which incubations were done with different concentrations of CaCl_2 . As with added NaCl, the liberation of uronic acid runs parallel with the loss of total carbohydrate from fibre.

DISCUSSION

Jansen, MacDonnell & Jang (1945) found that Mg^{++} ions inhibited the action of PG on pectic acid, and McColloch & Kertesz (1948) stated that Cu^{++} and Ca^{++} were inhibitory. In the present investigation the removal of calcium from leaf fibre was found to increase the attack on the pectic substances by PG.

The oxalic acid content of whole tobacco leaves has been given by Vickery & Pucher (1933) as about 2% of the dry matter, of which 80–90% is in a form insoluble in hot water, probably calcium oxalate. Ridgway (1916) observed crystals of calcium oxalate in both fresh and cured leaves. Holden & Tracey (1950) found that calcium oxalate was present in the deposit of chloroplasts and starch that is obtained on centrifuging 'sap' squeezed out from leaves after mincing. The calcium oxalate content of tobacco-leaf fibre was found to be about 1.5% of the dry matter assuming all the oxalic acid to be combined with calcium. Some plants, e.g. members of the Cucurbitaceae, contain large amounts of calcium carbonate in the leaves (Holden, 1948); however, little or none of the calcium of tobacco leaves is in this form. Calcium phosphate also does not appear to be present in any significant amount, as the inorganic phosphorus in tobacco fibre is only about 0.1% of the dry matter. The greater part of the calcium is probably combined with pectic substances, but some may be combined with protein or other substances.

Devaux (1901) showed that the insoluble pectic substances of the middle lamella and cell wall were able to fix metallic cations, and that these could be exchanged for each other; alkali-metal ions were able to displace calcium and other alkaline-earth cations and the reverse process readily occurred. He pointed out (1904) that this phenomenon was similar to that found with soils. McGeorge (1932), who was apparently unaware of Devaux's work, observed that dried alfalfa was able to exchange cations. He did not state the compound responsible, though in an earlier paper (McGeorge, 1931) he described cation exchange in 'lignins' from various sources. Wasserman (1949) showed that cations were adsorbed on the alginic acid present in the tissues of brown algae. In a recent paper Mattson, Eriksson, Vahtras & Williams (1949) commented on the high cation-exchange capacity of the pectin in the root membrane. Olsen (1948) found that some of the calcium could be displaced from beech-leaf tissue by

other cations, but that much of the calcium is present as oxalate in these leaves, and is not exchangeable.

It is not possible to remove all the calcium from fibre even with repeated extractions with ammonium acetate; however, subsequent extraction with dilute hydrochloric acid will remove all but traces. Extraction with salt does not remove all the oxalic acid, but the calcium remaining in the fibre is still in excess of the oxalic acid present. Some of the calcium combined with the pectin appears to be more firmly bound than the rest. Only after removal of this firmly bound calcium can pectic acid be extracted from fibre simply by neutralization. It may be that this calcium is forming cross-linkages between the polyuronide chains, as suggested by Bonner (1936), and when these are broken an increase in the solubility of the polyuronide might be expected.

No attempt has been made to distinguish between 'protopectin' or 'pectose' of the cell-wall and middle-lamella pectic substance, as it is probable that there is a smooth transition between them. The state of the pectic substances in fibre is not necessarily the same as in intact leaves. For example, additional calcium might be combined with the pectin in fibre since, if soluble calcium is present in the tissue, it could be taken up to replace the methoxyl groups removed by pectin-esterase action proceeding during preparation of the fibre. It is clear that part of the pectic substance in leaf fibre is not attacked by PG until the fibre has received treatments which remove calcium. However, in addition to calcium removal, other changes such as depolymerization may be brought about which make the substrate more easily attacked.

When calcium is added back to decalcified fibre, the calcium is taken up rapidly from solution. Fibre which has taken up the maximum possible calcium is scarcely attacked by PG even with prolonged incubation. The fact that only a small amount more of carbohydrate is released by PG from milled fibre than from minced shows that it is the state of combination of the pectic substances rather than their inaccessibility which influences the extent of the enzyme action.

Extracts made with ammonium oxalate or by incubation of fibre with PG contain more carbohydrate than is accounted for as polyuronide. This carbohydrate may have several origins: (1) Starch and its breakdown products inadequately removed before treatment of the fibre. (2) Other polysaccharides held entangled in structures whose integrity depends on polyuronide. (3) Part at least of the uronide may exist in the fibre in a form of combination with other sugar residues which remains stable even in solution.

The first will be discussed later (Holden & Tracey 1950), and the second has already been discussed (Holden *et al.* 1950). The third possibility has been

discussed by others (Hirst & Jones, 1939; Speiser, Eddy & Hills, 1945). In the present investigation, no separation of polyuronide from other polysaccharides has been attempted and no additional evidence is provided for or against the linkage of pectic acid with other cell-wall polysaccharides.

Purified PG causes disintegration of plant tissue, an effect which is usually ascribed to 'protopectinase' action (Beaven & Brown, 1949). There is the possibility that the preparation contains protopectinase in addition to PG. Alternatively, it may not be necessary to postulate the existence of an enzyme causing maceration of tissues distinct from pectin esterase and from PG. In leaf tissue, where pectic substances occur in combination with calcium, pectin esterase and PG do not cause complete disintegration without calcium removal, and this may be connected with other changes such as depolymerization. On the other hand, there appears little evidence that protopectin is more highly polymerized than soluble pectin. Since the nature of

protopectin is not clearly known, it is not possible at this stage to define the nature of protopectinase action.

SUMMARY

1. Tobacco-leaf fibre incubated at pH 4.5 several times with fresh lots of purified polygalacturonase loses about half of its polyuronide.

2. Milling the fibre increases the rate of release of carbohydrate from fibre with polygalacturonase and also increases slightly the total amount liberated.

3. Decalcification of the fibre by cation exchange with concentrated salt solutions, and by extraction with acid, increases the rate of liberation of carbohydrate by polygalacturonase, up to 95 % of the polyuronide present being released.

4. Sodium chloride in concentrations greater than 0.05 M inhibits the action of polygalacturonase on fibre, but concentrations less than 0.01 M activate. Calcium chloride is inhibitory, except in concentrations below about 0.001 M when using decalcified fibre.

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