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

5. UNFRACTIONATED FUNGAL ENZYMES

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Other papers in this series have described the effect on leaf tissue of snail digestive juice, purified polygalacturonase, and mammalian pancreatic and salivary enzymes (Holden, Pirie & Tracey, 1950; Holden, 1950; Holden & Tracey, 1950b). A number of preparations of fungal origin, known to contain carbohydrases, have been tested in the hope of finding one as effective as snail digestive juice in disintegrating leaf tissue but without some of its disadvantages. It was also hoped to find preparations in which the enzymes that attack leaf-fibre components were present in a different ratio from those in snail digestive juice, so that some substrates might be preferentially attacked.

Several commercial fungal enzyme preparations are available such as the Pectinols (Rohm and Haas Co.) which are used to facilitate filtration and clarification of fruit juices. Pectinols A and 100D have been investigated by Fish & Dustman (1945) for enzymic activity on known substrates. They found that Pectinol A, in addition to having pectin esterase and polygalacturonase activity, was able to split starch, sucrose, maltose and inulin, but not xylan. Another of the pectinols and extracts from two *Aspergillus* spp. were used by Hohl (1948) for macerating plant tissues for microscopic study. Emsweller (1944) used Clarase (Takamine Laboratory, Clifton N.J.) and extracts from a number of fungi, and Chayen (1949) used an extract from *Penicillium digitatum* for the same purpose. Hirst & Jones (1948) found that Pectinol 10M hydrolysed the galactomannan of carob-seed gum (from *Ceratonia siliqua*) into D-mannose, and also degraded ivory-nut mannan A. Pectinol 100D and D were used by Isbell & Frush (1944) for preparing D-galacturonic acid from pectin. Lineweaver, Jang & Jansen (1949) used Pectinols 45AP and 46AP as starting materials for the preparation of purified polygalacturonase. Pigman (1943) tested various commercial enzyme preparations for invertase and inulase activity. Takadiastase has been widely used in a great variety of work.

MATERIALS AND METHODS

The preparation of tobacco-leaf fibre and the analytical methods used have been described by Holden *et al.* (1950).

Enzyme preparations used

Pectinol 10 M, Pectinol A and Enzyme 19 AP (Rohm and Haas Co., Philadelphia) and a pectin-splitting enzyme '400-4' (Takamine Laboratory, Clifton N.J.). The solid was suspended in water, the solution centrifuged to remove insoluble material and then dialysed to lower the reducing-sugar content. Cellophan membranes were weakened by the concentration usually used (5% w/v); solutions were therefore dialysed in collodion sacs.

Preparations from Botrytis cinerea Pers. ex Fr., Aspergillus aureus Nakazawa, Penicillium expansum Link and Penicillium digitatum Sacc. The moulds were grown by inoculating moist bran with a spore suspension and incubating for 4-6 days at 25°. CHCl₃ was added, the bran dried at 37° and ground in a hammer mill. Enzyme solutions were prepared by suspending 10 g. dry bran in 100 ml. water. After standing for 1 hr. the extract was squeezed out from the bran, centrifuged and dialysed. Cellophan membranes could be used for dialysis of all except the *A. aureus* extract, for which collodion was necessary.

Takadiastase (undiluted, Parke, Davis and Co.). The solution was centrifuged and used without dialysis, as the reducing-sugar content was low.

Determination of enzyme activities on defined substrates

Cellulose. Cellulase activity on cellophan and sodium carboxymethylcellulose was determined by the methods described by Holden & Tracey (1950a).

Pectin. Pectin-esterase activity was determined as previously described (Holden, 1946), except that the incubation time was increased to 1 hr. or longer as the activity was low compared with extracts from leaf fibre, and a more acid buffer was used. The tests were done at pH 5.0 with acetate buffer present, as McColloch & Kertesz (1947) found that fungal pectin esterase had a lower pH optimum than that of plants. This was confirmed in the present investigation.

Pectic acid. Polygalacturonase activity of the various preparations was compared by determining the amount of reducing sugar produced from pectic acid. A comparison of the initial rate of formation of reducing sugar was made by the method of Jansen & MacDonnell (1945). For the figures given in Table 1, 25 mg. lots of pectic substance (78% polyuronide, prepared by enzymic demethylation) were incubated with the various enzyme preparations in the presence of 0.017 M-sodium acetate buffer (pH 4.5) for 20 hr. at room temperature in a total volume of 6 ml. and reducing sugar determined.

Starch. Amylase activity was determined by incubating 25 mg. lots of soluble starch with the enzyme preparations

Table 1. *Action of fungal enzymes on defined substrates*

(For conditions of incubation and analytical procedures, see also text, p. 426.)

Enzyme preparation	Percentage split in 20 hr.					
	Starch (25 mg. in 6 ml., 0.005 ml. enzyme solution, pH 4.5)	Pectic acid (25 mg. in 6 ml., 0.05 ml. enzyme solution, pH 4.5)	Haemoglobin (20 mg. in 6 ml., 0.05 ml. enzyme solution)		Inulin (20 mg. in 6 ml., 0.5 ml. enzyme solution, pH 5)	Cellulose (100 mg. in 6 ml., 1.0 ml. enzyme solution, pH 5)
			pH 4.5	pH 7.5		
Pectinol 10M	45	42*	20	27	20	2
Pectinol A	3	20	4	4	3	1
Enzyme 19AP	31	11	19	12	45	10
Takamine enzyme 400-4	11	37	23*	7	5	3
<i>Botrytis cinerea</i>	10% (w/v)	2	5	35	50	1
<i>A. aureus</i>	bran	23	11	7	5	5
<i>P. expansum</i>	extract	6	8	16	1	1
<i>P. digitatum</i>	(dialysed)	<1	16	10	5	2
Takadiastase	41*	8	48	46	<1	<1
Snail digestive juice	8	2	<1	<1	2	40

* With 1/5 the amount of enzyme solution.

in the presence of 0.017M-sodium acetate buffer (pH 4.5) and 0.17M-NaCl in a volume of 6 ml. at room temperature for 20 hr. The reducing sugar was determined by the hypiodite method, and in calculating the percentage split, the reducing sugar was assumed to be maltose (Table 1).

Inulin. 20 mg. lots of inulin were incubated with enzyme preparations in the presence of 0.033M-sodium acetate buffer (pH 5) in a volume of 6 ml. at room temperature for 20 hr. Reducing sugar was determined by the Hanes (1929) modification of the Hagedorn and Jensen method (Table 1). The activity on a grass levan, a levan from *Bacillus subtilis* and on irisin B was tested in the same way.

Xylan. 25 mg. lots of a crude preparation of maize xylan (polypentose content 60%) were incubated with enzyme preparations in the presence of 0.033M-sodium acetate buffer (pH 5) in a volume of 6 ml. and pentose determined by the method of Tracey (1950).

Proteins. (a) *Haemoglobin.* The activity of the preparations was tested at pH 4.5 and 7.5 on denatured haemoglobin in urea according to Anson (1938). For the former tests, the pH of the solution was adjusted with HCl. 20 mg. lots of haemoglobin (British Drug Houses Ltd.) were incubated with enzyme preparations in a total volume of 6 ml. for 20 hr. at room temperature. 6 ml. 10% (w/v) Trichloroacetic acid solution were added and the N still precipitable was determined (Table 1).

(b) *Gelatin.* Gelatinase activity was determined by measuring the time taken to liquefy a 5% gelatin gel at room temperature, and then repeating the determination with the amounts of enzymes adjusted so that liquefaction took place at approximately the same time.

General. The volume of enzyme used for a particular substrate was such that not more than half the substrate was split in 20 hr. with the most active preparation.

EXPERIMENTS AND RESULTS

ACTION OF ENZYMES ON DEFINED SUBSTRATES

From Table 1 it is clear that any differences in the action of the various preparations on fibre are likely to be due to differences in relative amounts of the enzymes and not to

their presence or absence. Polygalacturonase and cellulase, which may be expected to have most effect on the cell-wall constituents of fibre, vary widely in amount. The cellulase activity of the takadiastase, which was mentioned by Macfarlane & Rimington (1944), was only detectable using cellophan as substrate after several days' incubation. Activity on sodium carboxymethylcellulose, measured by reduction of viscosity, was readily detectable. The dialysed extract of *A. aureus* had cellulase activity on cellophan of the same order on a dry-weight basis as undialysed snail digestive juice, while the activity of dialysed 19AP was higher than this. Undialysed snail digestive juice is used as a basis for comparison, as dialysis did not lead to removal of much material and sometimes led to loss of activity (Holden & Tracey, 1950a). Dialysis of fungal extracts led to little loss of cellulase activity.

As leaf fibre has such high pectin-esterase activity, the addition of pectin esterase in enzyme solutions is of little importance particularly as the pectin-esterase activity of the fungal extracts is relatively low. A detailed comparison of the activity of the different preparations on pectin was therefore not made. *Botrytis cinerea* extract had the highest activity and 19AP and takadiastase the lowest. Pectinol 10M had the highest polygalacturonase activity and 1 ml. of a 5% (w/v) solution after dialysis hydrolysed pectic acid at about the same rate as 0.2 mg. of the purified enzyme. Except for that from *P. digitatum*, all the preparations were active on starch. This preparation showed low activity on inulin, as also did takadiastase. All preparations had some activity on the other fructosans tested as substrates, but these results are not given in Table 1, as it was not possible to repeat the determinations a sufficient number of times owing to the limited amount of material available. Activity on the crude xylan preparation was shown by all the preparations, 19AP and *A. aureus* extract being the most active. The most active preparation on haemoglobin was the Takamine pectin-splitting enzyme 400-4; takadiastase and *Botrytis cinerea* extract also had high activity. Pectinol A and *A. aureus* extract had low protease activity; snail digestive juice had no detectable activity. All the preparations caused liquefaction of gelatin, and pieces of vellum

Table 2. *Liberation of carbohydrate from tobacco-leaf fibre in one incubation with dialysed fungal extracts*

(0.5 g. lots fibre + 2 ml. 0.2M-sodium acetate buffer (pH 4.5) + 2 ml. enzyme solution + 4 ml., water. Incubated 24 hr. at 35°.)

Extract of	Dry matter content of extract added (mg.)	Carbohydrate liberated (mg./100 mg. dry fibre)		
		Fibre neutralized only	Fibre treated with trypsin	Fibre treated with trypsin then with polygalacturonase
<i>Penicillium digitatum</i>	5.0	10.0	14.1	9.1
<i>Botrytis cinerea</i>	5.8	17.3	18.4	7.8
<i>P. expansum</i>	5.0	18.1	20.5	7.2
<i>A. aureus</i>	4.4	30.0	34.0	15.7

were partially disintegrated when in contact with enzyme preparations overnight; snail digestive juice did not have this effect. The protease activity of snail digestive juice was also determined by the method of Tracey (1948), and was found to be less than that of tobacco-leaf sap. 1 ml. of the snail enzyme had an activity of <0.05 unit compared with a solution (5 mg. N/l.) of papain (British Drug Houses Ltd.) which had 0.42 unit/ml.

STUDY OF THE RELEASE OF CARBOHYDRATE FROM TOBACCO-LEAF FIBRE

Pretreatments of fibre

Neutralization. Fibre was neutralized before use to bring about pectin-esterase action (Holden, 1945), as removal of methoxyl groups from pectin is necessary for polygalacturonase action. There is also less pH change on subsequent incubation with enzymes when fibre has been previously neutralized.

Fig. 1. Effect of decalcification on liberation of carbohydrate by *A. aureus* extract from tobacco-leaf fibre. Portions of fibre (known dry-matter contents approx. 50 mg.) + 0.5 ml. 0.2M-sodium acetate buffer (pH 4.5) + 1 ml. dialysed *A. aureus* extract + 1.5 ml. water. Incubated for 1.5, 4.5 and 16 hr. at 38°. For decalcification procedures see Holden (1950).

Removal of potential substrates. All experiments were done on fibre from which starch had been removed by incubation with saliva or trypsin (Holden & Tracey, 1950b). For some experiments pectic substances were removed by incubation with purified polygalacturonase (Holden, 1950). Table 2

compares the amount of carbohydrate liberated in one incubation with dialysed fungal extracts from fibres pre-treated with different enzymes. After treatment with polygalacturonase there was a marked reduction in the amount of carbohydrate liberated on incubation with fungal extracts.

Decalcification. As with snail digestive juice and purified polygalacturonase, decalcification (by exchange of Ca for univalent cations and by acid extraction) prior to incubation with fungal enzyme preparations had a marked effect. Fig. 1 shows that the rate of liberation and the amount of carbohydrate released from fibre by *A. aureus* extract is increased.

Fig. 2. Effect of pH on liberation of carbohydrate from tobacco-leaf fibre by *A. aureus* extract and by snail digestive juice. 0.5 g. lots of trypsin-treated, partly decalcified fibre (dry matter 15.6%) + 1 ml. buffer solution (either 0.2M-sodium acetate or 0.2M-sodium phosphate) + 1 ml. dialysed *A. aureus* extract or 0.25 ml. snail digestive juice + 4 ml. water. Incubated 20 hr. at 37°.

Conditions of incubation

The optimum pH for carbohydrate liberation from fibre by fungal extracts was between pH 4.5 and 5. Fig. 2 compares the effect of pH on carbohydrate liberation by *A. aureus* extract and snail digestive juice. At pH 4.5 the

snail enzyme is only about half as active as at higher pH's. There was little change in pH during incubation with the fungal extract, but with snail digestive juice at pH's above 6 there was a marked fall.

The optimum pH for polygalacturonase action of snail digestive juice is at about 4.5, the same as for fungal polygalacturonase, but the enzyme is active at higher pH's than the fungal enzyme. The effect of pH on the cellulase activity, on cellophan, of snail digestive juice and *A. aureus* extract is shown in Fig. 3. The snail digestive juice has a sharp pH optimum at 5.5, while *A. aureus* has a less marked optimum at about pH 5, and is more active at more acid pH's than the snail cellulase.

Fig. 3. Effect of pH on cellulase activity of *A. aureus* extract and snail digestive juice on cellophan. Weighed pieces of cellophan (approx. 80 mg. dry matter) + 1 ml. buffer solution (0.2M-phosphate $\otimes$ $\oplus$, acetate $\times$ +, citrate $**$) + enzyme and water to give total volume 4 ml. Incubations with *A. aureus* extract: 2 ml. dialysed 10% (w/v) bran extract for 48 hr. at 35°. Incubations with snail digestive juice: 0.3 ml. snail enzyme for 24 hr. at 35°.

Effects on fibre

Table 3 shows the amounts of carbohydrate and N liberated from starch-free partly decalcified fibre with the various enzyme preparations including snail digestive juice. On a dry-weight basis the dialysed *A. aureus* extract and Enzyme 19AP are more active than snail digestive juice in removing carbohydrate from fibre. These two preparations and Pectinol 10M had the greatest disintegrating action on the fibre. The microscopic appearance of fibre residues after incubations with *A. aureus* or 19AP is very similar to that of fibre incubated with snail digestive juice (Holden *et al.* 1950). The other preparations had some effect on the texture of the fibre; takadiastase and *P. expansum* had the least. Takamine enzyme 400-4 removed the most N from the fibre, and this preparation was also the most active on haemoglobin. In spite of its low polygalacturonase activity, 19AP removed most of the polyuronide from the fibre.

Fig. 4 compares the amount of carbohydrate liberated from fibre by different amounts of snail digestive juice and *A. aureus* extract, and shows that the latter is about twice as active, weight for weight, as snail digestive juice.

The liberation of N by *A. aureus* extract was studied in more detail, since this is the most useful preparation for carbohydrate removal. Under all the conditions used the amount of N brought out from the fibre by enzyme action

did not exceed 15% of the N in the fibre, and was often less. At pH values between 4 and 8 there was little difference in the amount of N removed. Incubation in the presence of 0.02M-thioglycolic acid increased slightly the amount of N removed, but also increased the amount of N in the extracts from fibre incubated without enzyme. The increased amount of N in the control is due to the activation of the protease already present in the fibre (Tracey, 1948). Boiling the fibre before incubating with enzyme did not lead to increased N liberation, neither did decalcification. Incubation of fibre with trypsin at pH 7 will remove up to 80%

Fig. 4. Liberation of carbohydrate from tobacco-leaf fibre with different amounts of *A. aureus* extract and snail digestive juice. 0.5 g. lots of starch-free fibre (dry matter 22.9%) + 1 ml. buffer solution (sodium phosphate pH 6.5) for snail digestive juice, and sodium acetate (pH 4.5) for *A. aureus* extract + water and different amounts of enzyme solutions to give a total volume of 6 ml. Controls without enzymes at both pH's. Incubated at 35° for 18 hr.

of the N (Holden & Tracey, 1950b); therefore it is not due to the substrate being unavailable that such a small amount of N is liberated by *A. aureus*.

DISCUSSION

Enzyme preparations are available which have effects similar to those of snail digestive juice in disintegrating leaf fibre and releasing carbohydrate. They have the advantage that less dry matter has to be added to achieve the same result. An extract from *A. aureus* is the preparation that appears the most useful of those tested. It is twice as active on a dry-weight basis as snail digestive juice in liberating carbohydrate from fibre. It has higher activity on starch, pectic acid and inulin, and about the same activity on cellulose as snail digestive juice. The protease activity is low, though higher than that of snail digestive juice.

Decalcification of fibre before treatment with enzymes has a marked effect on the release of carbohydrate, but does not increase the amount of

Table 3. *Carbohydrate and nitrogen liberation from tobacco-leaf fibre by fungal and other enzyme preparations*

(0.5 g. lots of saliva-treated, partly decalcified fibre (dry matter, 17.4%; polyuronide, 20.2%; N, 3.55% and Ca, 0.67% dry matter) + 1 ml. 0.2M-sodium acetate buffer (pH 4.5) (1 ml. 0.2M-sodium phosphate (pH 6.5) for snail digestive juice) + water and enzyme solution to give volume of 4 ml. Three incubations for 24 hr. each at 35°. Residues dried, weighed and polyuronide and N determined.)

Enzyme used	Vol. of enzyme solution added	Dry-matter content of added enzyme preparation (mg.)	Carbohydrate in 1st extract (mg.)/100 mg. dry fibre	Reducing sugar as percentage of total carbohydrate in 1st extract	Carbohydrate in three extracts (mg.)/100 mg. dry fibre	Loss in wt. of fibre (mg./100 mg.)
Pectinol 10M	1 ml. 5% (w/v) solution (dialysed)	5.7	30.2	58	45.8	51.0
Pectinol A		3.0	26.4	—	40.5	37.0
Enzyme 19AP		3.4	48.1	59	62.4	63.3
Takamine enzyme 400-4		11.0	27.5	31	37.7	35.5
<i>Botrytis cinerea</i>	2 ml. 10% (w/v) bran extract (dialysed)	5.6	25.6	39	38.6	33.8
<i>A. aureus</i>		2.4	40.3	54	61.0	59.3
<i>P. expansum</i>		5.8	25.8	37	36.5	29.3
<i>P. digitatum</i>		4.6	22.6	44	36.0	35.4
Takadiastase	2 ml. 1% (w/v) suspension (centrifuged)	19.0	16.8	—	23.4	23.9
Snail digestive juice	Contents of 1 crop diluted to 1 ml.	23.3	36.2	41	57.7	57.5
Trypsin	1 ml. 1% (w/v) suspension (centrifuged)	8.0	2.7	—	5.3	11.2
Control	No enzyme	—	1.2	—	2.4	1.8

Polyuronide		Nitrogen		mg. N/100 mg. dry fibre residue
mg./100 mg. dry fibre residue	Percentage of original removed	mg./100 mg. dry fibre in three extracts	Percentage of original removed	
Pectinol 10M	5.4	87	0.74	5.02
Pectinol A	8.1	73	0.76	3.81
Enzyme 19AP	7.7	86	1.23	—
Takamine enzyme 400-4	8.0	75	1.30	3.00
<i>Botrytis cinerea</i>	13.2	56	1.02	3.71
<i>A. aureus</i>	9.7	78	0.66	6.44
<i>P. expansum</i>	11.3	60	1.09	3.58
<i>P. digitatum</i>	14.2	55	0.68	4.45
Takadiastase	15.5	42	1.01	3.40
Snail digestive juice	7.8	84	—	9.40*
Trypsin	21.9	0	0.58	3.43
Control	20.8	0	0.32	3.05

* High figure due to precipitation of N from the snail digestive juice.

nitrogen liberated. It therefore appears that intact protein is not released when the fibre framework is removed.

SUMMARY

1. The effects on tobacco-leaf fibre of some enzyme preparations of fungal origin have been investigated and compared with those of snail digestive juice.

2. The enzymic activities of the preparations on a number of polysaccharides (cellulose, pectic acid, starch and inulin) and on haemoglobin and gelatin were determined.

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Cellulase from Leaves and Roots of Tobacco

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In the course of work on the enzymic breakdown of leaf fibre (Holden, Pirie & Tracey, 1950), it was found that leaf sap apparently has a weak cellulase activity. There are few reports of the existence of cellulase in the higher plants and all are concerned with its presence in germinating seeds or young seedlings (Newcombe, 1899; cf. Lüers & Volkamer, 1928; Grüss, 1928; Lüers & Malsch, 1929). Some preliminary observations on the presence of a cellulase in the tobacco plant and parts of some other plants are presented in this paper, because further work on the subject is not contemplated at the moment.

MATERIALS

Glasshouse-grown *Nicotiana tabacum* var. White Burley was used for much of the work, but parts of other plants listed later were also used. Tobacco plants were divided into leaves, stems and roots. The midribs were cut from the leaves, care being taken that as little as possible of the lamina was left attached to them. Roots were washed in running water until free from obvious contamination, and dried by pressing in cloth. The four batches of material were then minced separately in a domestic meat mincer and the sap squeezed out by hand through madapolam. The saps were centrifuged for 15 min. at about 1500 g and stored with CHCl_3 at 5°.

Snail digestive juice was got from the crops of starved snails as described by Bawden & Pirie (1946).

METHOD

Active plant extracts reduce the viscosity of a solution of sodium carboxymethylcellulose at pH 5. The rate of viscosity reduction is of the same order as that got with an

equal volume of a 1:100,000 dilution of *Helix* digestive juice (undiluted crop contents). This amount is too small for convenient measurement of its activity by methods other than viscosity reduction. Hyaluronidase activity is commonly measured (e.g. Madinaveitia & Quibell, 1940) by determining the time taken for half the viscosity increment due to the substrate to be lost. For accurate measurement by this method, it is necessary to know the viscosity at zero time and the viscosity at the completion of the action. Since relatively large volumes of sap must be used to get measurable viscosity reductions, and since the sap contains a substance or substances that react with the substrate, the zero time viscosity can only be measured by back extrapolation, which is not accurate owing to the rapid change in viscosity early in the reaction. The final viscosity cannot be measured since it would take a considerable time for the action to be completed, and during this time precipitation from the sap may occur. Consequently, the viscosity of the enzyme-substrate mixture was measured after 5 and 15 min. action and the rate of change of viscosity calculated. The value $(\log VI_5 - \log VI_{15})$ was used as a measure of the rate of change of viscosity (VI_5 and VI_{15} are the viscosity increments at 5 and 15 min.; the viscosity increment being taken as relative viscosity - 1). If the rate of viscosity reduction, produced by differing amounts of diluted *Helix* enzyme under the conditions specified below, is plotted against the amount of enzyme, a straight line is got when the amounts of *Helix* enzyme are in the range 0.002-0.01 μl . undiluted crop contents. With varying amounts of active tobacco sap the curve is smooth, and almost linear at the beginning. The variation from linearity may be due to variation in the amount of salts added in the sap and to variations in the amount of substrate combining with the material in the sap. It is possible, using such a curve, to make approximate estimations of the amount of enzyme present. The viscosity