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Carbohydrases of the Rumen Ciliate *Epidinium ecaudatum* (Crawley)

HYDROLYSIS OF PLANT HEMICELLULOSE FRACTIONS AND β-LINKED GLUCOSE POLYMERS

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1. Cell-free extracts from *Epidinium ecaudatum* (Crawley) hydrolysed the three hemicellulose fractions of pasture plants, but at different rates. 2. All of the constituent monosaccharides are released from the hemicellulose fractions, galactose and uronic acids being liberated at much slower rates than pentoses. 3. An arabinofuranosidase, which removes arabinose from highly branched arabinoxylan before the xylan chain can be hydrolysed, was isolated free from other pentosanases. 4. A xylanase hydrolysing xylan (by random cleavage) and xylodextrins of degree of polymerization (D.P.) > 3 to xylotriose and xylobiose was isolated free from other pentosanases. 5. A separate xylodextrinase hydrolysing (by random cleavage) xylodextrins of D.P. > 2 to xylobiose and xylose was also obtained; this enzyme did not hydrolyse xylan or xylobiose and the original extracts themselves possessed very weak xylobiase activity. 6. The epidinial extracts hydrolysed laminaribiose, laminarin, lichenin and cellodextrins of D.P. < 7 rapidly, cellobiose and gentiobiose slowly but cellulose not at all. 7. Polysaccharide glucose associated with plant linear B hemicellulose was liberated with cellobiose and possibly laminaribiose as intermediates. 8. The cellodextrinase hydrolysed cellopentaose initially to cellobiose plus cellotriose and is a distinctly different enzyme from the xylanase and xylodextrinase. 9. Extracts from *Entodinium* species and *Eremoplastron bovis* also hydrolysed all three types of plant hemicellose.

The presence of hemicellulase activity in rumen oligotrich ciliates was first demonstrated with cell extracts from *Epidinium ecaudatum* (Crawley) (Bailey, Clarke & Wright, 1962). Since then this type of activity has been shown to be present in similar extracts of the rumen oligotrichs *Entodinium* spp. (Bailey & Clarke, 1963*a*), *Eremoplastron bovis* (Bailey & Clarke, 1963*b*) and *Polyplastron multivesiculatum* (Abou-Akkada, Eadie & Howard, 1963). With *Epidinium ecaudatum* the previous work (Bailey *et al.* 1962) was confined largely to a study of the hydrolysis of the easily prepared, water-soluble, highly branched wheat-flour arabinoxylan and a commercially available, water-insoluble xylan. The actual hydrolysis of the complete plant hemicellulose complex, which would be the natural substrate for the organisms, was only briefly examined. Grass and clover hemicellulose consist, however, of xylans containing uronic acid (D-glucuronic acid, where identified) as well as D-xylose and L-arabinose, together with a highly branched polymer fraction rich in D-galactose and uronic acid in addition to these pentoses. By use of the procedure developed by Gaillard (1961) for the fractionation of plant hemicellulose B, purified samples of the various hemicellulose fractions have been prepared from several pasture plants. A detailed study of the action of the epidinial enzyme complex on these fractions has been made. The pentosanases in the complex have been fractionated and their mode of action on pentosans has been established.

Cellulase activity in extracts of rumen oligotrichs was originally reported by Hungate (1943) but the presence of a true cellulase in cell-free extracts of these organisms has not been confirmed. Although the *Entodinium* and *Eremoplastron* extracts hydrolysed cellodextrins they did not hydrolyse cotton-wool cellulose (Bailey & Clarke, 1963*a*,*b*), and the *Polyplastron* extracts hydrolysed only part of a filter-paper cellulose preparation (Abou-Akkada

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et al. 1963). As the epidinial extracts prepared for the present work hydrolysed cellodextrins their action on various β-linked glucose polymers has also been investigated.

MATERIALS AND METHODS

Carbohydrates

Oligosaccharides. Cellobiose and gentiobiose were from L. Light and Co. Ltd. Laminaribiose was isolated by aqueous ethanol fractionation, on a charcoal–Celite column, of a partial acid hydrolysate of laminarin. β-(1→4)-Linked xylodextrins (xylobiose–xylopentaose) and cellodextrins (cellotriose–cellopentaose) were isolated by the same fractionation procedure from partial hydrolysates of commercial xylan and cellulose respectively. Paper-chromatographic analysis showed that all of the oligosaccharides contained not more than traces (less than 0·5% by visual estimation) of contaminant sugars. Xylopentaitol and cellopentaitol were prepared by reducing the pentaoses with sodium borohydride as described by Bailey & Roberton (1962).

Plant hemicellulose fractions. Linear A, linear B and branched B fractions were prepared (B. D. E. Gaillard, unpublished work) from leaf plus stem tissue of ryegrass (*Lolium perenne*) and red clover (*Trifolium pratense*) and stem tissue from soya bean (*Glycine max*). Linear A (hemicellulose A) is a water-insoluble β-(1→4)-linked xylan containing uronic acid and little or no arabinose. Linear B is a β-(1→4)-linked xylan with less uronic acid and more arabinose side chains than linear A; it is also more soluble and probably of shorter chain length than linear A. Branched B is a highly branched polymer rich in galactose and uronic acid as well as in xylose and arabinose and is water-soluble when isolated (linear B + branched B = hemicellulose B). The detailed monosaccharide composition of the fractions is given in Table 1.

Other polysaccharides. Water-insoluble commercial xylan was from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., and contained 7% of arabinose. Soluble wheat-flour arabino-xylan was prepared from unbleached wheat flour by the method of Howard (1957) and contained 13% of arabinose. Cellulose powder was prepared from absorbent cotton wool by acid degradation, followed by ball-milling (Clarke, 1964). Laminarin and lichenin were from L. Light and Co. Ltd. Rhodymenan [water-soluble linear xylan containing β-(1→3)- and β-(1→4)-links; Howard, 1957] was kindly supplied by Professor B. H. Howard.

Protozoal extracts

Epidinium ecaudatum (Crawley). Suspensions of this organism were isolated from rumen liquor obtained from cows fed on fresh red clover (*T. pratense*) as described by Bailey *et al.* (1962). A total of 25 g. (wet wt.) of centrifuged protozoa was prepared and deep-frozen until required. Half of the preparation was disrupted by grinding as a thick paste with water and Ballotini beads for a few minutes (E_1). The other half (E_2) was disintegrated in water in a Teflon tissue disintegrator (Bailey, 1958). Both extracts were centrifuged twice at 20000*g* for 30 min., dialysed against distilled water and freeze-dried to yield E_1, 110 mg. (9·94% N) and E_2, 152 mg. (14·0% N). Both extracts hydrolysed the same range of carbohydrates at similar rates.

Other oligotrichs. The aqueous extracts of mixed *Entodinium* species and of *Eremoplastron bovis* were those used in the previously reported studies (Bailey & Clarke, 1963*a*,*b*).

Enzyme digests

Digests in the present work were prepared in citrate buffer (0·1 M, pH 6·0; Bailey *et al.* 1962) except where stated. Linear A and B fractions, lichenin and laminarin were prepared in this buffer by first dissolving in dilute alkali followed by neutralization with citric acid to pH 6·0. Branched B fractions could be dissolved directly in the buffer or in water. Satisfactory resolution on paper chromatograms of the complex mixtures of sugars present in the concentrated dialysis digests (see below) required removal of the buffer salts (Bio-Deminrolit; The Permutit Co. Ltd.) with a consequent loss of uronic acid fragments. Separate digests containing the branched B fractions were therefore prepared in water instead of buffer; for the linear fractions buffer ions were removed by the dialysis technique

Table 1. *Monosaccharide composition of plant hemicellulose fractions*

—, Not detected.

Species	Fraction	Xylose	Arabinose	Galactose	Uronic acid	Glucose
		(% of anhydro-monosaccharide)				
L. perenne (grass)	Linear A	85·2	12·9	—	1·9	—
	Linear B	82·6	13·9	—	4·5	(19·0)†
	Branched B	63·1	23·8	7·7	5·4	—
T. pratense (clover)	Linear A	96·7	Trace	—	3·3	—
	Linear B	88·2	10·8	—	1·0	(11·6)†
	Branched B	17·3	27·6	34·5	20·6	—
G. max (soya bean)	Linear A	98·8	—	—	1·2	—
	Branched B*	3·4	24·0	34·3	24·6	6·9

* Also contains 6·8% of rhamnose.

† Glucose in these fractions is probably present as a separate neutral glucan (B. D. E. Gaillard, unpublished work). Concentrations of other sugars in these fractions are calculated on a glucose-free basis.

as described below. Control digests showed that the rates of hydrolysis of all the hemicellulose fractions in citrate buffer and in water were essentially similar with the exception of grass branched B which was hydrolysed much faster in the buffer (2–3 × water rate). All incubations were at 39° under toluene and appropriate controls showed that neither protozoal extract produced free sugars when incubated without substrate and that none of the substrates was hydrolysed when incubated alone under these conditions.

Qualitative digests. These contained, unless stated otherwise, extract (1mg.), carbohydrate (2mg.) and citrate buffer (0·1ml.) or were a larger-scale version of this composition. After 0 and 24hr. incubation or at intervals up to 24hr. they were analysed by paper chromatography (50 or 100 μl. per spot) for liberated sugars.

Quantitative digests. For comparison of the rates of hydrolysis of plant hemicellulose fractions these contained extract (2mg.), polysaccharide (20mg.) and citrate buffer (2ml.). At intervals during incubation duplicate portions (50 μl.) were removed for the measurement of total reducing sugars and paper-chromatographic analysis. Other quantitative digests contained the same ratio of enzyme to buffer but different amounts of substrate and are detailed in the appropriate experimental section.

Dialysis digests. To remove the early products of hydrolyses from the enzyme activity, digests with concurrent dialysis were carried out by the following technique. A simple dialysis chamber, consisting of a length of glass tubing (7·0cm. × 3cm. diam.) closed at one end with a bung and at the other with a piece of dialysis membrane attached with a rubber band, was constructed. The membrane was cut from opened-out $\frac{20}{32}$ Viscose dialysis tubing (Viscose Dept., Union Carbide International Co., U.S.A.) and pre-soaked in water. Control experiments showed that, at 39°, about half of the xylopentaose in a 1% solution (2ml.) passed through this membrane in about 10–15min. In use the membrane end was immersed just below the surface of the external solution (citrate buffer or water, 10ml. in a 50ml. beaker) and digest solution (2ml.) placed in the chamber. During incubation both solutions were gently stirred with magnetic stirrers and at intervals (1–6, 10min. intervals; later intervals, 30min.) the external solution was removed and replaced by fresh solution. Each diffusate, desalted if buffer, was concentrated to a small volume for paper-chromatographic analysis. Unless stated otherwise the digest solution was of the same composition as the quantitative digests. Dialysate digests of uronic acid-free polymers were carried out in citrate buffer but those containing branched B polymer contained water. To follow uronic acid liberation in the water-insoluble linear fractions these were dissolved (linear B) or dispersed (linear A) in the buffer, via solution in alkali (see above), and dialysed without enzyme for 1hr. at 39° to remove buffer salts. Enzyme extract was then added and the dialysis continued against water.

Carbohydrate analyses

Paper chromatography. Papers were usually developed with solvent (a) (ethyl acetate–water–pyridine, 2:2:1, by vol.) and sugars located with aniline hydrogen phosphate (Howard, 1957). Other solvents used in identifying liberated sugars were: (b) the modified version of solvent (a) devised by Malpress & Hytton (1958) for the separation of higher oligosaccharides, (c) ethanol–butan-1-ol–water–aq. ammonia (sp.gr. 0·88) (4:1:4·9:0·1, by vol.), (d) butan-1-ol–pyridine–water (5:3:2, by vol.) and (e) ethyl acetate–acetic acid–formic acid–water (9:1·5:0·5:2, by vol.). Spray reagents used were $AgNO_3$ (Trevelyan, Procter & Harrison, 1950), diphenylamine–aniline (Bailey & Bourne, 1960) and benzylamine–ninhydrin (Bayly & Bourne, 1953).

Reducing sugars. These were measured by the microcuprimetric method of Nelson (1944) with xylose or glucose, which give almost the same colour per 100 μg. of sugar, as standards.

RESULTS

Hydrolysis of oligosaccharides by Epidinium ecaudatum *extracts*

Disaccharides. Qualitative digests showed that both extracts (E_1 and E_2) hydrolysed xylobiose, cellobiose and gentiobiose slowly but hydrolysed laminaribiose much more rapidly. The hydrolysis rates of these disaccharides were measured by following the increase in reducing sugars in digests containing extract (E_2, 2mg.), disaccharide (5mg.) and citrate buffer (2ml.). The rates of hydrolysis, expressed as mg. of disaccharide hydrolysed/hr./mg. of extract nitrogen, when not more than 35% hydrolysis had occurred, were: xylobiose 0·29, cellobiose 0·21, gentiobiose 0·20, laminaribiose 1·11. Similar digests containing xylose or glucose showed that both extracts were without action on these monosaccharides.

Xylodextrins and cellodextrins. Standard qualitative digests showed that both extracts hydrolysed the triose–to–pentaose members of these two series at much faster initial rates than the corresponding disaccharides.

The rates of hydrolysis of xylotriose, cellotriose and the two sugars together were followed by measuring at intervals the increase in reducing power in digests containing E_1 (1mg.), trisaccharide (5mg.; 10mg. with combined sugars) and citrate buffer (1ml.). The rates of increase in reducing power, calculated as glucose, are shown in Fig. 1. In the early stages the increase in reducing power corresponded to the cleavage of the non-reducing end glycosidic link in 6·7 and 36·1mg. of xylotriose and cellotriose/hr./mg. of enzyme nitrogen respectively.

The mode of hydrolysis of the pentasaccharides was investigated in digests containing extract (E_2, 1mg.), pentaose (10mg.) and citrate buffer (1ml.), which were analysed by paper chromatography at intervals during incubation. Early stages of the xylopentaose digests showed spots corresponding to all of the members of the series xylose to xylotetraose. As incubation continued xylose and xylobiose accumulated whereas first xylotetraose and then xylotriose disappeared. Cellopentaose digests showed cellotriose in the early stages and,

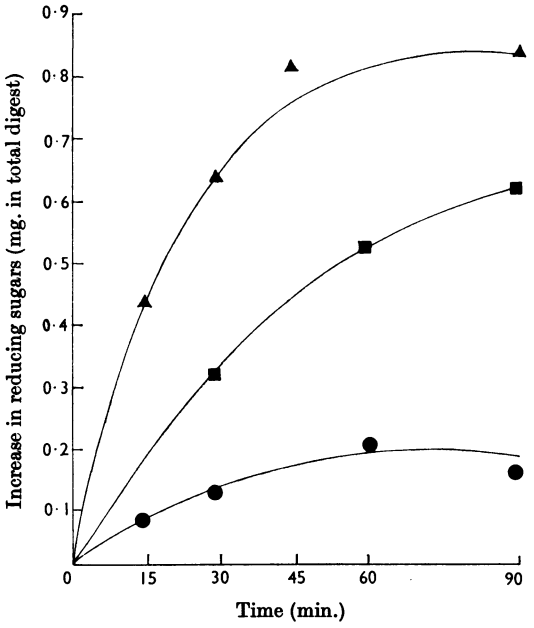

Fig. 1. Hydrolysis of trisaccharides by epidinial extracts. Digests contained extract (E_1, 1mg.), trisaccharide (5mg.; 10mg. with combined sugars) in citrate buffer (1ml.). Portions (50μl.) were analysed for reducing sugars at intervals. ●, Xylotriose; ■, cellotriose; ▲, xylotriose+cellotriose.

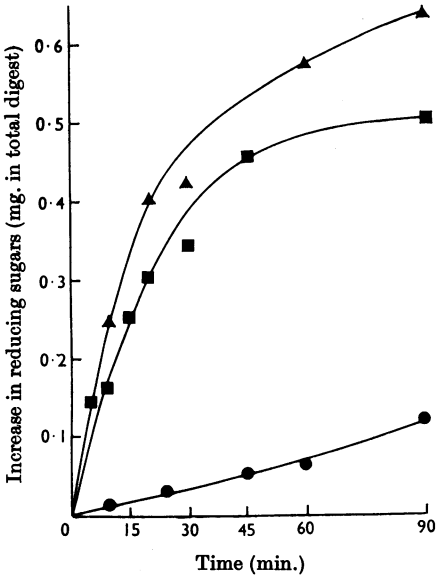

Fig. 2. Hydrolysis of pentasaccharides by epidinial extract. Digests contained extract (E_1, 1·5mg.), pentasaccharide (15mg., 30mg. combined sugars) in citrate buffer (3ml.). Portions (50μl.) were analysed for reducing sugars at intervals. ●, Xylopentaose; ■, cellopentaose; ▲, xylopentaose+cellopentaose.

at later stages, glucose; there was no sign at any stage of cellotetraose. A similar digest containing xylopentaitol showed the early release of xylose, xylobiose and xylotriose but not of xylotetraose (aniline hydrogen phosphate spray) with an early release also of a non-reducing component corresponding to xylotetraitol (silver nitrate spray). In a cellopentaitol digest cellobiose and a non-reducing component corresponding to cellotriitol but not cellotriose were the only sugars detected in the early stages.

The rates of hydrolysis of xylopentaose, cellopentaose and the two sugars combined were similarly measured in digests containing E_1 (1·5mg.), pentasaccharide (15mg.; 30mg. with combined sugars) and citrate buffer (3ml.). The rates of increase in reducing power, calculated as glucose, are shown in Fig. 2. In the early stages the increase in reducing power corresponded to a possible hydrolysis of between 22·5 and 12·0mg. (values calculated on the basis of the removal of xylose and xylobiose respectively from the non-reducing end of the pentaose) of xylopentaose/hr./mg. of extract nitrogen. For cellopentaose the increase in reducing power corresponded to the hydrolysis of 168mg. of cellopentaose/hr./mg. of nitrogen, here calculated on the basis of the removal of cellobiose units from the non-reducing end of the pentaose.

Hydrolysis of polysaccharides by Epidinium ecaudatum *extracts*

Hydrolysis of branched arabino-xylan, linear xylans and rhodymenan. Standard qualitative digests containing E_1 or E_2 and wheat-flour arabino-xylan analysed at intervals by paper chromatography showed, in agreement with previous results (Bailey *et al.* 1962), a steady accumulation of xylose, xylobiose and arabinose with only traces of xylodextrins of higher degree of polymerization (D.P.). Digests containing the almost unbranched linear A xylan from soya bean and a much higher ratio of substrate to enzyme (E_1, 3mg.; polysaccharide, 20mg.; buffer, 2ml.) showed a different pattern of oligosaccharide release. In this case the chromatograms showed an early release of xylose, xylobiose and xylo-triose to -pentaose, all of which accumulated during the first 7hr. After 24hr. only xylose and xylobiose were found. At all times the intensities of the xylo-tetraose and -pentaose spots were much weaker than those of the lower homologues.

The tetra- and penta-ose were not detected alone before the appearance of the sugars of lower D.P. A digest containing grass linear B xylan (10mg.), extract (E_2, 1mg.) and buffer (1ml.) was incubated for 5hr. and analysed by paper chromatography with solvent (d). Chromatograms of the digest showed, in addition to xylose and xylodextrins of D.P. 2–5, definite spots corresponding to xylo-hexaose, -heptaose and -octaose.

The liberation of xylodextrins of D.P. > 3 as intermediates in xylan hydrolysis was more clearly demonstrated in dialysis digests. In such digests containing wheat-flour arabino-xylan or commercial xylan the first four diffusates showed the presence of large amounts of arabinose, xylose, xylobiose and xylotriose. Paper chromatograms of later diffusates showed in addition to the above four compounds intense spots corresponding to xylo-tetraose, -pentaose and possibly homologues of higher D.P. In similar digests containing the soya-bean linear A xylan, however, all diffusates (10min. onwards) showed on the chromatograms intense spots corresponding to all members of the series xylose to xylopentaose. A dialysis digest of red-clover linear A xylan in water gave additional spots (see Table 2) whose movement and staining properties were those expected for uronic acid compounds.

Rhodymenan, in qualitative and dialysis digests, showed a steady release of the series xylose to xylopentaose. Xylotetraose and xylodextrins of higher D.P. were not detected before the appearance of the lower homologues. Rhodymenabiose [xylosyl-β-(1→3)-xylose] was not detected at any stage, and it is probable that β-(1→3)-xylosidic linkages were also hydrolysed; rhodymenabiose itself was not available for testing against the extract.

Hydrolysis of pasture plant hemicellulose fractions. The rates of hydrolysis of the three hemicellulose fractions (linear A, linear B and branched B) from ryegrass and red clover were compared by measuring the release of reducing sugars from these polysaccharides in standard quantitative digests. Results obtained are shown in Fig. 3. Chromatographic analysis of the linear A and B digests showed a rapid release and accumulation of xylose and xylobiose with xylotriose and xylodextrins of higher D.P. present only in the early stages. Arabinose was released early and accumulated to amounts expected from the arabinose content of each polymer. With the branched B fractions there was also a rapid release of arabinose, a slower release of xylose and a very much slower release of uronic acid compounds. Free galactose was not detected in the grass branched B digest but it was released very slowly, together with a possible hexose disaccharide, in the clover branched B digest. In both of these digests components

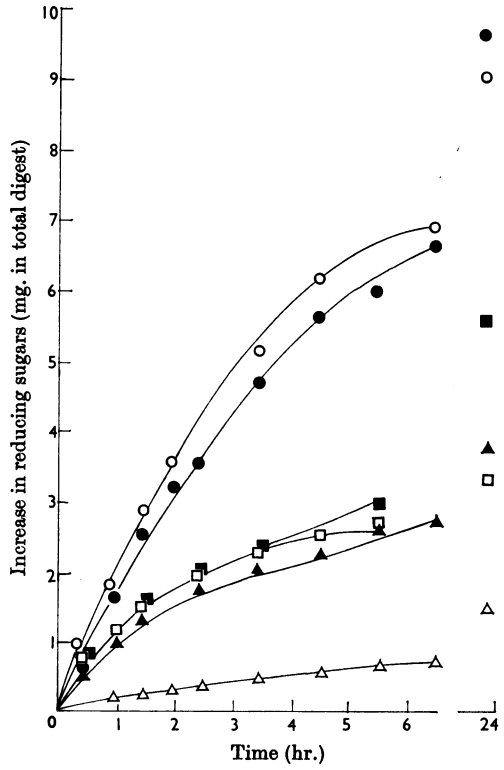

Fig. 3. Hydrolysis of pasture-plant hemicellulose fractions by epidinial extracts. Standard quantitative digests contained extract (E_1, 2mg.), polysaccharide (20mg.) and citrate buffer (2ml.). Portions (50μl.) were analysed for reducing sugars at intervals. Grass: ■, linear A; ●, linear B; ▲, branched B. Clover: □, linear A; ○, linear B; △, branched B.

chromatographically identical with xylobiose and xylotriose were detected in the early stages of the digestion. Digests containing grass, clover and soya-bean branched B respectively and a higher ratio of enzyme to substrate (E_1, 10mg.; polysaccharide, 20mg.; water, 2ml.) were similarly analysed at intervals by paper chromatography. Results were essentially similar to the quantitative digests with galactose and uronic acid compounds being released more slowly than the pentoses. In these digests galactose was liberated from all three branched B fractions and the glucose in the soya-bean branched B fraction was also released.

The hydrolysis of the grass branched B fraction, in addition to the soya-bean linear A hydrolysis already mentioned, was also followed in standard dialysis digests under the conditions which avoid the need for desalting. In addition clover branched B fraction was hydrolysed in a dialysis digest

Table 2. *Sugars detected in dialysis digests of plant hemicellulose fractions and epidinial extracts*

Standard dialysis digests (for composition see the text) were incubated in water. Diffusates were changed at 10min. intervals up to 1hr. (nos. 1–6), then at 30min. intervals (no. 7 onwards), concentrated and analysed by paper chromatography. Chromatogram spot intensities: weak, just clearly visible; moderate, intensity equal to 10–30μg. of sugar; intense, intensity equal to > 50μg. of sugar. Abbreviations: aldur, uronic acid compounds; X_{2-5} etc., β-(1→4)-linked xylodextrins.

Plant fraction	Paper-chromatographic identity of liberated sugars	Spot intensity in diffusates (no.)
Soya-bean linear A	Xyl, X_{2-5}	Moderate (2–4), intense (5–8)
	Aldur	Weak (7–8)
Red-clover linear A	Xyl, $X_{2,3}$	Moderate (2), intense (3–7)
	$X_{4,5}$	Weak (3–7)
	Aldur	Weak (3–6), moderate (7)
Ryegrass branched B	Ara	Moderate (1–6), intense (7–13)
	Xyl, $X_{2,3}$	Moderate (7–11), weak (12–13)
	$X_{4,5}$	Weak (7–12)
	Aldur	Weak (10–13)
Red-clover branched B*	Ara	Intense (1–8)
	Xyl, X_2	Moderate (2–8)
	Gal	Weak (2–7), moderate (8)
	Aldur	Weak (2–6), moderate (7, 8)

* Dialysis digest containing higher ratio (1:2) of enzyme to substrate.

containing a higher ratio of enzyme to polysaccharide (E_2, 10mg.; polysaccharide, 20mg.; water, 2ml.). Details of the identities and rates of liberation of the various sugars detected in the diffusates of these digests are summarized in Table 2. A separate dialysis digest of grass branched B fraction was carried out, with repeated changes of external solution, for 24hr. The residual polysaccharide was analysed for galactose and pentose monosaccharides, other than that present in aldobiouronic acid, by hydrolysis in 1N-sulphuric acid at 100° for 2hr. followed by quantitative paper chromatography (Wilson, 1959). The ratio of galactose to pentoses in the hydrolysate of the residue was 1:3·2 compared with 1:9·0 in the original polymer (both ratios uncorrected for aldobiouronic acid). The pentoses in the hydrolysate of the residual polysaccharide consisted of approximately 40% of arabinose and 60% of xylose.

Hydrolysis of glucose polysaccharides. Digests containing hemicellulose linear B fractions showed a rapid release of the glucose present in these fractions. Chromatograms of these digests showed, in addition to glucose, the presence of two possible hexose disaccharides. One of these, disaccharide A, gave a moderately intense spot, but the other, disaccharide B, gave a spot of much weaker intensity and was detected only in the early stages of the digests. Disaccharide A showed the presence of a reducing aldohexose unit (aniline hydrogen phosphate spray) and a (1→4)-glycosidic link (diphenylamine–aniline). It was chromatographically identical with cellobiose in all solvents but different from sophorose, laminaribiose, gentiobiose (solvents a, c, with benzylamine–ninhydrin, d and e) and maltose (solvent e). Disaccharide A was therefore tentatively identified as cellobiose. Disaccharide B, in the solvents in which it separated from xylobiose, had a similar rate of movement to laminaribiose but was not further investigated.

Laminarin in a standard large-scale qualitative digest showed on chromatograms a steady accumulation of glucose but no sign of laminaribiose or of laminaridextrins of higher D.P. In similar digests containing lichenin chromatograms showed, in addition to accumulating glucose, intense spots tentatively identified as cellobiose and cellotriose plus a much weaker spot corresponding to laminaribiose. No detectable sugars were liberated by either extract in standard qualitative digests containing cellulose.

Fractionation of epidinial extracts

Fractionation on Sephadex G-100. Extract E_2 (20mg. in citrate buffer, 10ml.) was fractionated on a column (25cm. × 3cm. diam.) of Sephadex G-100 (Pharmacia, Uppsala, Sweden) which had been well washed with the buffer. The column was eluted with citrate buffer (200ml.) and fractions (10ml.) were collected. Each fraction was tested for activity against wheat-flour arabinoxylan, soya-bean linear A xylan, xylodextrins and cellodextrins in digests containing fraction (0·3ml.) and carbohydrate (1mg. in citrate buffer, 0·1ml.), which were incubated for 24hr. and analysed by paper chromatography for liberated sugars. The results obtained are listed in Table 3. The distribu-

Table 3. *Fractionation of epidinial hemicellulase on Sephadex G-100*

Activities were tested in digests containing fraction (0·3ml.) and carbohydrate (1mg. in citrate buffer, 0·1ml.) incubated for 24hr. and analysed by paper chromatography. Abbreviations: X_{3-5} etc., β-(1→4)-linked xylodextrins; $(C)_2$, cellobiose; $(C)_5$, cellopentaose.

Fraction	Carbohydrates hydrolysed	Carbohydrates not hydrolysed
1–5	None	All
6	Wheat-flour arabino-xylan (arabinose only liberated), X_{3-5}*	Linear A xylan, X_2, $(C)_2$
7–13	Wheat-flour arabino-xylan, X_{2-5}, linear A xylan, $(C)_{2-5}$	None
14–16	Linear A xylan,† $X_{4,5}$,† $(C)_5$	Wheat-flour arabino-xylan, $X_{2,3}$, $(C)_2$
17–20	None	All

* Random hydrolysis ultimately to xylobiose and xylose.
† Random hydrolysis ultimately to xylotriose and xylobiose ($X_5 \rightarrow X_2 + X_3$; $X_4 \rightarrow X_2$).

Table 4. *Fractionation of epidinial hemicellulase on diethylaminoethylcellulose*

For details of digests see Table 3. Abbreviations: as in Tables 2 and 3.

Fraction	Carbohydrates hydrolysed	Carbohydrates not hydrolysed
	(a) Eluent 0·01M-citrate (pH 6·0)	
1–9	None	All
10	Wheat-flour arabino-xylan (arabinose only liberated)	All others
11–18	Wheat-flour arabino-xylan (arabinose and †), linear A xylan,† $X_{4,5}$†	$X_{2,3}$, $(C)_2$, $(C)_5$
19–26	None	All
	(b) Eluent 0·05M-citrate (pH 6·0)	
27–36	None	All
37–40	X_{3-5},* $(C)_5$	Wheat-flour arabino-xylan, linear A xylan, X_2, $(C)_2$
41–46	None	All
	(c) Eluent 0·1M-citrate (pH 6·0)	
47–60	None	All

* Random hydrolysis ultimately to xylobiose and xylose.
† Random hydrolysis ultimately to xylotriose and xylobiose ($X_5 \rightarrow X_2 + X_3$; $X_4 \rightarrow X_2$).

tion of other glucosidases in the fractions was not investigated because of shortage of suitable substrates. In addition, a digest containing fraction 15 (2ml.) and soya-bean linear A xylan (5mg.) was incubated for 6hr. and analysed by paper chromatography with solvent (b). In this case xylodextrins of D.P. > 5, as well as the lower homologues, were found. The xylanase in tubes 14–16 hydrolysed xylopentaose to xylotriose plus xylobiose and xylotetraose to xylobiose only.

Fractionation on DEAE-cellulose. Fractions 7–13 (Table 3) were combined, dialysed against water, freeze-dried, dissolved in citrate buffer (0·01M, pH 6·0) and adsorbed on to a column (2cm. × 21cm.) of DEAE-cellulose (Cellex D; California Corp. Biochemical Research) previously washed with the same buffer. The column was eluted successively with 0·01M-, 0·05M- and 0·1M-citrate buffer (pH 6·0, 200ml. each) and the fractions (10ml.) collected were tested against the same carbohydrates as in the Sephadex fractionation. The activities of the fractions are listed in Table 4. The xylodextrinase in tubes 37–40 hydrolysed xylopentaose via all its lower homologues to xylobiose plus xylose.

Hydrolysis of plant hemicellulose fractions by Entodinium *and* Eremoplastron bovis *extracts*

The activity of these two extracts was tested in qualitative digests containing extract (0·3ml.) and polysaccharide (2mg.) incubated for 24hr. Chromatograms showed that both extracts liberated large amounts of xylobiose and xylotriose plus smaller amounts of xylose, xylodextrins (D.P. > 3) and uronic acid compounds from soya-bean linear fraction. Both extracts had a much weaker

activity against grass and clover branched B fractions, trace amounts only of spots corresponding to xylose, xylobiose, xylotriose and arabinose being detected on the papers.

DISCUSSION

From studies with branched arabino-xylan, Bailey *et al.* (1962) concluded that epidinial hemicellulase action involved prior removal of arabinofuranose single unit side chains followed by slower stepwise (exoenzyme) removal of terminal xylobiose units from the main xylan chain. With this type of substrate the preliminary removal of arabinose units, by a separate enzyme, has now been confirmed. Chromatography on DEAE-cellulose yielded a fraction (tube 10, Table 4) which liberated only arabinose from arabino-xylan and the Sephadex column yielded xylanase fractions (tubes 14–16, Table 3) which had no action on the arabino-xylan.

In the light of present experiments it is clear that hydrolysis of the xylan chain is by random cleavage of the chain (endoenzyme) rather than stepwise removal of xylobiose units (exoenzyme). This is most clearly shown by the dialysis digest experiment on soya-bean linear A when xylodextrins of D.P. > 3 were rapidly produced in large amounts. Results from digests containing xylopentaose or its alcohol also indicated random cleavage of xylodextrins of D.P. > 2. When the arabino-xylan was hydrolysed in a dialysis digest, although there was a rapid early release of arabinose and xylodextrins of D.P. < 3 the liberation of dextrins of higher D.P. was very much slower than with the soya-bean linear A xylan. As arabinose must first be removed from arabino-xylan to permit xylanase action it is possible that the rate of arabinose removal has some modifying effect on the random hydrolysis of the xylan chain.

The type of dialysis digest used in the present work proved extremely useful in obtaining an understanding of the mode of action of the enzyme system before actual fractionations were attempted. It was evident, however, that care was necessary in choosing a dialysis membrane which permitted the rapid passage of tetrasaccharides and higher saccharides.

Fractionation studies on the extracts separated two classes of xylan-hydrolysing enzymes: a xylodextrinase hydrolysing xylo-triose, -tetraose and -pentaose, but not xylans or xylobiose, by random cleavage was obtained free from other pentosanases from the DEAE-cellulose column (tubes 37–40, Table 4), whereas Sephadex fractionation yielded a xylanase (tubes 14–16, Table 3) free from the xylodextrinase and arabinofuranosidase. This latter enzyme hydrolysed linear xylan and xylodextrins of D.P. > 3 to mixtures of xylotriose and xylobiose and liberated xylodextrins of D.P. > 5 as intermediates in the hydrolysis of soya-bean linear xylan. Although the xylanase could be a mixture of a true xylanase and a second xylodextrinase it was not further resolved by the second fractionation, on DEAE-cellulose, and is most probably a single endoenzyme hydrolysing β-(1→4)-xylose polymers of D.P. > 3. The combined action of the xylanase and xylodextrinase on xylodextrins probably explains why dextrins of D.P. > 3 were not previously detected before the appearance of the lower homologues although they would have been expected to do so with simple endoxylanase action.

No separation of xylobiase activity was obtained by the fractionation procedures. In any case the xylobiase activity in the extracts was very much lower than the xylodextrinase activity, even when compared with the hydrolysis rate of xylotriose. Water extracts of epidinia contain amylase but not maltase (Bailey, 1958) and it is possible that most of the xylobiase also may not be extracted by water. In a study of rumen bacterial pentosanases Howard, Jones & Purdom (1960) isolated a xylanase with similar properties to the one described here together with a second enzyme which they called a xylobiase as it hydrolysed xylodextrins of D.P. > 2 at a slower rate than xylobiose. The xylodextrinase obtained in the present work does not hydrolyse xylobiose (tubes 36–40, Table 4) and is clearly not a xylobiase.

The results from the fractionation of the epidinial extract on Sephadex (Table 3) suggest that the xylodextrinase is a molecule of larger volume than the xylanase. This observation is similar to that of Pettersson & Porath (1963), who found in a study of the enzymes in the cellulolytic complex from *Polyporus versicolor* that the β-glucosidase was a molecule of larger volume than the actual cellulase. Sephadex fractionation also indicated that the arabinofuranosidase, which removes directly the smallest fragment from the xylan molecule, preceded both the xylodextrinase and xylanase and must have the largest volume of these three enzymes.

The epidinial hemicellulase complex is able to hydrolyse all three pasture-plant hemicellulose fractions and liberate at different rates all of the constituent monosaccharides. Presumably carbohydrases other than the ones actually examined in the present work form part of this complex and are involved in the hydrolysis of the plant fractions. The epidinial hemicellulase is essentially similar to rumen bacterial hemicellulases in activity against grass and clover hemicellulose. Both linear B fractions were hydrolysed the most rapidly and at the same rates. Both linear A fractions were

hydrolysed at similar rates up to 6·5hr. but, though hydrolysis of clover linear A had almost ceased after this time, the hydrolysis of grass linear A continued steadily for at least 24hr. The grass branched B fraction was, however, hydrolysed at a much faster rate than clover branched B. The present results are in agreement with Gaillard's (1962) findings that grass hemicellulose has a higher digestibility than clover hemicellulose and the different hydrolysis rates of the fractions can be explained in terms of uronic acid or galactose units interfering with access of the pentosanases to the pentose portions of the molecules. The general pattern of activity of epidinial hemicellulase against plant hemicellulose appears to apply more generally to the oligotrich protozoa. Thus though *Entodinium* and *Eremoplastron* extracts were also able to hydrolyse plant linear hemicellulose fractions quite readily they had a much weaker hydrolytic action on the branched B hemicellulose fractions.

Plant linear hemicellulose fractions contain β-(1→4)-linked xylose but the nature of the possible xylose–xylose links in the clover and grass branched B fractions is unknown. The detection of components chromatographically identical with xylobiose and xylotriose in branched B digests suggests, however, that much of the xylose in these fractions is β-(1→4)-linked. These xylose oligosaccharides have been examined only on paper chromatograms and the possibility that they contain arabinose–xylose oligosaccharides of the type obtained in the enzymolysis of wheat arabinoxylan by Goldschmid & Perlin (1963) cannot be excluded.

It has not been possible to demonstrate activity towards cellulose but only towards cellodextrins which are not natural substrates. The cellodextrinase activity present in the extracts could represent one of three classes of enzyme: (*a*) part of a true cellulase complex which is not extracted or is inactivated; (*b*) part of an enzyme complex hydrolysing the β-(1→3)–β-(1→4)-linked glucans which occur in small amounts in plants; (*c*) the xylodextrinase itself acting on cellodextrins, since β-(1→4)-linked xylodextrins may be regarded as cellodextrins lacking the 6-CH_2·OH groups. This third hypothesis is unlikely as the cellodextrinase activity is now seen to be separate from the xylodextrinase activity for the following reasons. First, the hydrolysis rate of the total extract on cellobiose + xylotriose and on cellopentaose + xylopentaose was in each case close to the sum of the rates on the separate sugars. Secondly, cellopentaose is hydrolysed only by the removal of cellobiose from its non-reducing end whereas the xylodextrinase present hydrolyses xylopentaose by random cleavage. Thirdly, fractionation on DEAE-cellulose clearly separated the cellodextrinase activity from the xylanase, which does hydrolyse xylopentaose to xylobiose and xylotriose, and associated it with the random-cleaving xylodextrinase.

Laminaribiose, laminarin [β-(1→3)-glucan] and lichenin [β-(1→3)–β-(1→4)-glucan] in addition to cellodextrins were readily hydrolysed by the epidinial extracts. For this reason the cellodextrinase activity could be part of an enzyme complex hydrolysing β-(1→3)–β-(1→4)-glucans. The linear B fractions used contained 10–20% of glucose (see Table 1) believed to be present as a separate, contaminating glucan (B. D. E. Gaillard, unpublished work). With *L. perenne*, for example, this linear B glucose corresponded to approximately 0·5% (dry wt.) of glucan in the original plant tissue. The extracts rapidly liberated this linear B glucose and disaccharides chromatographically identical with cellobiose and possibly laminaribiose were detected as intermediates.

In view of these results it is clear that evidence for the presence of true cellulase in rumen oligotrich protozoa still requires the demonstration of the hydrolysis of undegraded cellulose by cell-free extracts.

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