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# DIGESTION OF POLYSACCHARIDE CONSTITUENTS OF TROPICAL PASTURE HERBAGE IN THE BOVINE RUMEN

## PART IV. THE HYDROLYSIS OF HEMICELLULOSES FROM SPEAR GRASS BY CELL-FREE ENZYME SYSTEMS FROM RUMEN FLUID

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ABSTRACT

Hemicelluloses have been isolated from spear grass (*Heteropogon contortus*), before and after digestion in the rumen, and separated into linear and branched fractions. Similar fractions have also been obtained from a pasture sample and from the faeces fibre of a steer fed on the same pasture. The rates of hydrolysis of all of these hemicellulose fractions have been determined in the presence of extracellular enzymes from rumen fluid and of enzymes liberated by disruption of rumen micro-organisms. The oligosaccharide products of such enzymic degradations have been partially identified. The results confirm that the incomplete digestion of hemicelluloses in the rumen is due to physical protection (*e.g.* by lignin), rather than to structural differences between different components of the hemicelluloses. There is no difference between rates of digestion of branched and linear hemicelluloses, and previous results which indicated such differences were probably caused by presence of a readily digested glucan in linear hemicellulose fractions.

## INTRODUCTION

Bailey and his co-workers have studied the action of cell-free hemicellulases from rumen bacteria and protozoa on hemicellulose fractions[1,2] of plant pasture. They showed that the relative rates of hydrolysis of the linear B and branched B polymers from rye grass (*L. perenne*) and red clover (*T. pratense*) were essentially similar for the hemicellulases from the protozoa and rumen bacteria. In all cases, the linear B fractions were hydrolysed more rapidly than the branched B fractions.

A number of workers have examined the oligosaccharide products of the hydrolysis of hemicelluloses with cell-free enzyme systems and intact organisms. Howard[3] incubated a highly purified, wheat-flour arabinoxylan with a suspension of rumen bacteria (from sheep) in the presence of toluene or chloroform, and arabinose, xylose, xylobiose [$\beta$-D-Xyl*p*-(1 → 4)-D-Xyl], xylotriose, and xylotetraose were detected on paper chromatography of the hydrolysate. No compounds of low mole-

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cular weight containing both arabinose and xylose were reported but, in a later paper[4], Howard reported the presence of various amounts of L-arabinose in the total, acid hydrolysate of fractions obtained by charcoal-column fractionation of the above polysaccharide after partial digestion by rumen bacteria. He concluded that the hydrolysis of the wheat-flour arabinoxylan proceeded by simultaneous removal of arabinose side-units and fragmentation of the main xylose chain. Pazur and his co-workers[5], using a frozen and thawed rumen-liquor extracted from an Angus bull and a xylan extracted from corn cobs, concluded that the oligosaccharides produced by hydrolysis fell into two groups. One included the $\beta$-(1 $\rightarrow$4)-linked D-xylose series while the other, present in smaller amounts, was suggested to be a mixed oligosaccharide series containing both arabinose and xylose. Dehority[6], using isolated rumen bacteria, showed the presence of oligosaccharides in the digestion of xylans. Walker and Hopwood[7] extracted and partially purified hemicellulases from mixed micro-organisms from sheep rumen. The cell-free enzyme was reported to catalyse the hydrolysis of hemicellulose to xylose, xylobiose, xylotriose, and higher oligosaccharides, together with glucose and arabinose. Abou-Akkada and his co-workers[8] showed that a cell-free extract from the rumen protozoa *Polyplastron multivesiculatum* readily hydrolysed wheat-flour arabinoxylan to mainly xylose and arabinose, and small amounts of xylobiose and other oligosaccharides. Bailey and his co-workers[1,2], using cell-free protozoal sources and linear and branched hemicellulose B polymers from grass and clover, identified the presence of a homologous series of oligosaccharides containing xylose only. These workers did not rule out the possibility that arabinose–xylose oligosaccharides were also present.

The presence of significant, extracellular carbohydrase activity in the rumen fluid has generally been discounted[3,9–13]. However, Gill and King[14] have demonstrated the presence of free cellulases in rumen fluid. Howard *et al.*[15] found that the clear supernatant obtained by centrifuging cultures of *Bacteroides amylogenes* and a strain tentatively assigned to the genus *Butyrivibrio* grown on a medium containing wheat-flour arabinoxylan showed a little enzyme activity. Traces of xylose, arabinose and oligosaccharides could be produced on incubation with wheat-flour arabinoxylan.

## EXPERIMENTAL

Optical rotations were measured with a Perkin–Elmer Model 141 polarimeter. Paper chromatography (p.c.) was carried out on Whatman No. 1 paper, using (*a*) ethyl acetate–pyridine–water (10.4:3), (*b*) butan-1-ol–ethanol–water (3:1:1), (*c*) butan-1-ol–pyridine–water (6:4:2.5), (*d*) propan-1-ol–ethyl acetate–water (14:2:7). Detection was effected with silver nitrate[16] and *p*-anisidine hydrochloride[17]. $R_{XYL}$ values of sugars refer to distances moved relative to D-xylose. Reducing sugars were measured by the Nelson method[18]. The degree of hydrolysis of the polysaccharides was calculated by equating the Nelson reducing-power of the hydrolysates with that of known amounts of xylose, and this method was used to obtain "conversion" values in Tables I and II. Concentrations of hemicellulose and oligosaccharides were

measured by the phenol–sulphuric acid procedure[19], using L-arabinose and D-xylose standards.

*Collection of rumen liquor, preparation of cell-free rumen fluid, and cell-free enzyme preparations.* — Rumen liquor from the steers was brought to the laboratory in thermos flasks (~2 h), strained through Terylene cloth, and centrifuged (20,000 *g*, 20 min). The supernatant was stored at −10° until required, and neither protozoa nor bacteria could be detected on microscopic examination. The mixed protozoal and bacterial pellet was washed twice with McIlvaine's buffer (0.1M, pH 6.6), then suspended in the same buffer (the final volume was equal to the original volume of rumen liquor), and stored at −10°. The cell-free preparations of enzyme were prepared by disrupting the cells by sonication for a total of 10 min at such intervals that the temperature did not rise above 12°. The sonicated cells were centrifuged at 20,000 *g* (30 min, 4°) and used immediately.

The sample fibre types were used as described earlier[20], namely (*a*) fresh, vegetative spear grass (*Heteropogon contortus*), (*b*) the same grass after digestion for 3 days within a Terylene bag in the rumen, (*c*) a stand of pasture which was predominantly spear grass (~75%), with no other significant, single species of grass or clover present, (*d*) fibre isolated from faeces of steers fed on pasture (*c*). Samples (*c*) and (*d*) are described as "intake fibre" and "faeces fibre" respectively. The isolation of hemicellulose B-fractions and branched and linear sub-fractions from each type of fibre has also been described previously[20].

*Preparation of hemicellulose solutions.* — Solutions (~1%) of hemicelluloses were prepared in McIlvaine's buffer by heating the solution in a boiling-water bath (20 min). The solutions on cooling were centrifuged (2500 r.p.m.), and the small proportion of undissolved material was discarded. The concentrations of these solutions were estimated by the phenol–sulphuric acid procedure[19].

*Enzyme digests.* — Unless otherwise stated, the volume ratio of cell-free enzyme solution or centrifuged rumen liquor to substrate solution was 1:9. Each digest was carried out in duplicate at 39° under toluene. Aliquots (50 μl) were removed at intervals up to 72 h, and reducing power was measured by the Nelson procedure[18]. Other samples (2 ml) were heated in a boiling-water bath (15 min), deionised by mixed resins [Amberlite IR-120($H^+$) and IR-45($HO^-$)], and examined by paper chromatography with solvent (*a*). The cell-free enzyme system (0.1 ml) was also incubated for 48 h with solutions (0.9 ml) containing L-arabinose, xylobiose, xylotriose, and xylotetraose (~1% with respect to each component). Examination by paper chromatography, using solvent system (*a*), showed no arabinoxylose oligosaccharides.

*Quantitative enzyme digest and identification of neutral oligosaccharides.* — A solution of spear-grass hemicellulose (8 g) in McIlvaine's Buffer (400 ml, 0.1M) was incubated with cell-free enzyme solution (90 ml) for 96 h and then centrifuged (3000 r.p.m., 10 min). The precipitate was washed with water and then ethanol, and dried *in vacuo* at 40° to constant weight (1.7 g). The increase in reducing power of the solution at this stage corresponded to 22% conversion into xylose. Part of the

supernatant (480 ml) was heated in a boiling-water bath (20 min), cooled, deionised with Amberlite IR-120($H^+$) and IR-45($HO^-$) resins, and concentrated to a syrup (4 g). Paper chromatography in solvent (*a*) indicated the presence of xylose, arabinose, and glucose, together with seven slower-moving components (**1**–**7**) having $R_{XYL}$ values of 0.58, 0.39, 0.38, 0.30, 0.22, 0.15, and 0.12, respectively. These components were subjected to a preliminary separation on Whatman No. 17 paper in solvent (*a*) (400 mg distributed over 35 cm; running time, 12 h). Components **1** and **4**–**7** were re-purified on Whatman 3MM paper, using solvent (*a*); for the last two components, the paper was eluted for 7 days. Components **2** and **3** were separated on Whatman 3MM paper, using solvent (*b*). Identifications were as follows.

**1** Xylobiose [β-D-Xyl*p*-(1→4)-D-Xyl] (9 mg), $[\alpha]_D^{23}$ −24.7° (*c* 0.9, water); lit.[21] $[\alpha]_D$ −25°; was homogeneous by paper chromatography in 3 solvents, showing $R_{XYL}$ 0.58 in solvent (*a*) (lit.[22] value 0.60), 0.60 in solvent (*b*), and 0.68 in solvent (*c*). After hydrolysis with 0.5M sulphuric acid at 100° for 7 h, xylose was the only product revealed by paper chromatography in solvent (*a*).

**2** Arabino-xylotriose (3 mg), $[\alpha]_D^{23}$ −70.0° (*c* 0.3, water), was homogeneous (p.c.), showing $R_{XYL}$ 0.39 in solvent (*a*), 0.60 in solvent (*b*), and 0.56 in solvent (*c*). Hydrolysis with 0.5M sulphuric acid at 100° for 4 h, followed by conversion of the hydrolysate into the acetylated alditols[23] and g.l.c., showed the presence of arabinose and xylose in the ratio of 1.0:2.8. After partial hydrolysis (20mM sulphuric acid at 100° for 20 min), p c. of the neutralised solution ($BaCO_3$), using solvent system (*a*), showed the presence of xylotriose and arabinose, together with traces of xylobiose and xylose.

**3** Cellobiose (3 mg), $[\alpha]_D^{23}$ +34° (*c* 0.3, water), was homogeneous (p.c.), showing $R_{XYL}$ 0.38 in solvent (*a*), 0.42 in solvent (*b*), and 0.56 in solvent (*c*), and was identical with authentic cellobiose. After hydrolysis with 0.5M sulphuric acid at 100° for 4 h and neutralisation, p.c. with solvent system (*a*) showed the presence of D-glucose only.

**4** Xylotriose (20 mg), $[\alpha]_D^{23}$ −61° (*c* 2, water) (lit.[21] $[\alpha]_D$ −60°), was homogeneous (p.c.), showing $R_{XYL}$ 0.30 in solvent (*a*) (lit.[22] value 0.30), 0.28 in solvent (*b*), and 0.41 in solvent (*c*). On hydrolysis with 0.5M sulphuric acid at 100° for 7 h, the oligosaccharide yielded D-xylose only [p.c., solvent (*a*)].

**5** Arabino-xylotetraose (7 mg), $[\alpha]_D^{23}$ −73.0° (*c* 0.7, water), was homogeneous (p c.), showing $R_{XYL}$ 0.22 in solvent (*a*), 0.13 in solvent (*b*), and 0.32 in solvent (*c*). Hydrolysis with 0.5M sulphuric acid at 100° for 4 h, with subsequent conversion of the hydrolysate into the acetylated alditols and g.l.c., showed the presence of arabinose and xylose in the ratio of 1.0:3.7. After partial hydrolysis (20mM $H_2SO_4$ at 100° for 20 min), p.c. of the neutralised solution ($BaCO_3$), using solvent system (*a*), showed the presence of xylotetraose and arabinose, together with traces of xylose, xylobiose, and xylotriose.

**6** Xylotetraose (8 mg), $[\alpha]_D^{23}$ −61.0° (*c* 0.8, water) (lit.[21] −60°), was homogeneous (p.c.), showing $R_{XYL}$ 0.15 in solvent (*a*) (lit.[22] 0.16) and 0.21 in solvents (*b*) and (*c*). After hydrolysis with 0.5M sulphuric acid at 100° for 7 h, the oligosaccharide yielded D-xylose only when examined by p.c. in solvent (*a*).

**7** Arabino-xylopentaose (6 mg), $[\alpha]_D^{23}$ −75.0°, was homogeneous (p.c.), showing

$R_{XYL}$ 0.12 in solvent (*a*) and 0.16 in solvents (*b*) and (*c*). Hydrolysis with 0.5M sulphuric acid at 100° for 4 h, with subsequent conversion of the hydrolysate into the acetylated alditols and g.l.c., showed the presence of arabinose and xylose in the ratio 1.0:4.7. After partial hydrolysis (20mM sulphuric acid at 100° for 20 min), p.c. indicated the presence of arabinose and a slow-moving component (assumed to be xylopentaose), together with traces of xylotetraose, xylotriose, xylobiose, and xylose.

*Further incubation of the cell-free enzyme digest.* — To a sample (10 ml) of the 96-h digest described above, a further volume (5 ml) of cell-free enzyme preparation was added. The incubation was terminated after a further 72 h, and the apparent conversion into xylose at this stage was 35%. The solution was deionised with a mixed bed of Amberlite IR-120($H^+$) and IR-45($HO^-$) resins, and examination by p.c. in solvent (*a*) indicated the presence of xylose, arabinose, glucose, xylobiose, and xylotriose, plus traces of the slower-moving oligosaccharides.

## DISCUSSION

We have considered the possibility that the resistance to rumen digestion of a part of the hemicellulose of spear grass is due to resistance of part of the hemicellulose molecule to enzymic attack on a structural or chemical basis[20]. This possibility now appears to be firmly discounted by the results given in Table I, which show that the total hemicellulose B, and also the branched and linear sub-fractions, are attacked by the cell-free preparation of rumen enzyme at the same rate, irrespective of whether the polysaccharides are obtained from fresh spear grass, or from the same grass digested for 3 days in the rumen. Similar results were obtained with hemicelluloses from a predominantly spear-grass pasture (intake fibre) in comparison with fibre from the faeces of steers fed on the same pasture (faeces fibre). We must conclude, there-

TABLE I

RATES OF DIGESTION OF HEMICELLULOSE FRACTIONS BY CELL-FREE RUMEN ENZYME-PREPARATIONS

| *Source* | *Hemicellulose* | *Apparent conversion into xylose* (%) | | |
|---|---|---|---|---|
| | | *18 h* | *40 h* | *72 h* |
| Spear grass | Total B fraction | 20.0 | 28.6 | 32.5 |
| | Linear | 15.6 | 24.0 | 27.6 |
| | Branched | 16.2 | 23.8 | 27.0 |
| Digested spear grass | Total B fraction | 17.2 | 26.5 | 31.0 |
| | Linear | 15.2 | 23.0 | 26.4 |
| | Branched | 16.2 | 26.0 | 29.4 |
| Intake fibre | Total B fraction | 15.6 | 23.0 | 27.0 |
| | Linear | 17.0 | 23.7 | 27.5 |
| | Branched | 13.5 | 18.7 | 23.4 |
| Faeces fibre | Total B fraction | 16.8 | 23.6 | 26.6 |
| | Linear | 15.2 | 22.2 | 25.9 |
| | Branched | 15.8 | 21.7 | 26.4 |

fore, that the part of the hemicellulose which resists digestion is equally as susceptible to enzymic attack as the original, total hemicellulose. This leaves us with the conclusion that the predominant cause of resistance to digestion of hemicelluloses in spear grass is physical protection, associated, for example, with lignin.

These results fully support the earlier, similar work by Bailey and his co-workers[1,2,24]. The earlier papers[1,2] by these workers showed that the cell-free preparations of enzyme from total rumen bacteria, and also from a single bacterial strain from the rumen and a protozoal strain, would rapidly and extensively convert isolated hemicelluloses of several temperate grasses and legumes into mono- and oligo-saccharides. Their later work[24] showed that hemicelluloses isolated from caecum and abomasum contents and from faeces were capable of further digestion by rumen enzymes (after delignification). In the latter study, the temperate pasture plants contained significant proportions of hemicellulose A (water-insoluble) and were probably less heavily lignified than the spear grass used in our work.

The results shown in Table I indicate no significant difference in rate of attack of the total rumen enzymes on branched and linear sub-fractions of the hemicellulose B. This result is in complete contrast to those of Bailey and his co-workers[1,2] which show a much higher rate of attack by both rumen bacterial and protozoal enzymes on the linear hemicellulose-B fractions from temperate clover and grass. The cause of this difference is not immediately evident. It seems unlikely that it is due to differences between digestion in sheep (used by Bailey) and cattle (used in our work), so that we should perhaps look for differences between the hemicelluloses of the tropical spear grass and the temperate rye grass (*Lolium perenne*, used by Bailey). No major, structural differences have been found between hemicelluloses of temperate and tropical grasses[25], and the arabinose–xylose ratios and uronic acid contents of our branched and linear fractions are quite similar to those reported for rye grass[1]. The rye-grass, linear B fractions, however, contained major quantities of glucose, "probably present as a separate neutral glucan". Since glucose was rapidly released in digestion of these fractions, it now seems quite likely that the apparently more-rapid digestion of the linear-B hemicelluloses[1,2] was due to the rapid digestion of a contaminating, neutral glucan in this fraction and that there is in fact little difference between the rate of rumen digestion of linear and branched hemicellulose fractions from any of the pastures studied to date.

Previous workers[3,9–15] have shown that the proportion of *extracellular* polysaccharase activity in the rumen is very small, and we have confirmed this with our system. Thus, Table II shows data for the digestion of hemicellulose fractions by cell-free rumen fluid. Comparison with the results in Table I, at the same hemicellulose concentration, suggests that extracellular hemicellulase activity in the rumen is at least an order of magnitude less than the intracellular activity which is released on disruption of cells of the rumen micro-organisms.

A comparison by paper chromatography of the products from action of extra- and intra-cellular rumen enzymes on hemicelluloses showed that there were no major differences in the visual patterns obtained. The first component to appear (after

TABLE II

DIGESTION OF SPEAR-GRASS HEMICELLULOSES BY CELL-FREE RUMEN FLUID

| *Hemicellulose* | *Apparent conversion into xylose* (%) | | |
|---|---|---|---|
| | *18 h* | *40 h* | *72 h* |
| Total hemicellulose B | 1.4 | 2.5 | 3.5 |
| Linear fraction | 1.3 | 2.3 | 3.3 |
| Branched fraction | 1.5 | 2.4 | 3.5 |

digestion for 6 h was glucose, together with cellobiose, the slower-moving oligosaccharides, and xylose and arabinose. After 72 h of digestion, the amounts of xylobiose, xylotriose, xylotetraose, arabinose, and xylose had increased relative to glucose. The cellobiose was only present in trace amounts. In each sample, a second series of oligosaccharides of mixed composition (containing xylose and arabinose) was also present. After digestion for 72 h, the branched fractions yielded more arabinose than xylose, whereas the linear fractions yielded approximately equal amounts of the two pentoses.

In a separate experiment, the intracellular enzyme preparation was diluted with an equal volume of McIllvaine's buffer and incubated for 48 h. Examination of the solution by paper chromatography showed no traces of reducing sugars, thus confirming that the glucose was produced from the hemicelluloses (or a glucan contaminant therein), and not from a component such as starch from the cell debris.

Seven oligosaccharides were isolated from action of rumen enzymes on the spear-grass hemicellulose in order to determine the types of linkage most resistant to enzyme attack. The oligosaccharides were tentatively identified by optical rotation, paper chromatography in various solvents, and complete and partial hydrolysis; the results are summarised in Table III. Incubation of arabinose with a mixture of xylobiose, xylotriose, and xylotetraose, in the presence of the same enzyme, yielded no arabinoxylose oligosaccharides, thus confirming that these originated from the hemicellulose and not by transglycosylation. Since we know[26] that the arabinose units in spear-grass hemicellulose are present as L-arabinofuranose groups attached to position 3 of the xylan backbone, the major uncertainty remaining in the structure of the arabinoxylose oligosaccharides is to determine which of the xylose residues carries the arabinose. This question will be the subject of further investigation. The relative yields of the xylose oligosaccharides indicate that the xylanases present are predominantly endo-enzymes and that they require an uninterrupted xylan chain of at least four xylose residues for hydrolysis to be rapid. Xylotetraose is rapidly attacked, since further treatment of the oligosaccharide products with the same enzyme preparation yielded mainly arabinose, xylose, xylobiose, and xylotriose, with only traces of higher oligosaccharides.

In addition to the xylan oligosaccharides, a glucose disaccharide chromatographically identical with cellobiose was also extracted and identified. It is assumed

that this accumulated as a result of the hydrolysis of a small glucose-containing fraction in the hemicellulose, possibly a degraded cellulose, or perhaps another glucan (*cf.* Ref. 27).

TABLE III

OLIGOSACCHARIDES FROM ACTION OF RUMEN ENZYMES ON SPEAR-GRASS HEMICELLULOSE

| | *Yield (mg)* | *Composition* | *Identification* |
|---|---|---|---|
| 1 | 9 | $Xyl_2$ | Xylobiose |
| 2 | 3 | Ara-$Xyl_3$ | Arabino-xylotriose |
| 3 | 3 | $Glc_2$ | Cellobiose |
| 4 | 20 | $Xyl_3$ | Xylotriose |
| 5 | 7 | Ara-$Xyl_4$ | Arabino-xylotetraose |
| 6 | 8 | $Xyl_4$ | Xylotetraose |
| 7 | 6 | Ara-$Xyl_5$ | Arabino-xylopentaose |

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