Degradation of hemicellulose and pectin by horse caecum contents

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1. Polysaccharide depolymerases and glycoside hydrolases involved in the breakdown of plant structural polysaccharides (hemicellulose and pectins) were monitored in three fractions of the liquid phase of horse caecum digesta: acellular fluid (AF), bacteria (B) and protozoa plus bacteria (PB).

2. Both bacteria and protozoa were found to be involved in the decomposition of pectic substances, with two enzymic activities: depolymerase (polygalacturonase, *EC* 3.2.1.15; and pectin lyase, *EC* 4.2.2.10) and esterase (pectinesterase, *EC* 3.1.1.11). The activity of the PB fraction was higher than that of B.

3. With hemicellulosic substrates, all three fractions showed a significant xylan endo-1,3-β-xylosidase (*EC* 3.2.1.32) activity. Mannan was hardly broken down.

4. Galactomannan and arabinogalactan were broken down more extensively by the PB fraction than by the B fraction. Glycosidase activities (xylan 1,4-β-xylosidase, *EC* 3.2.1.37 and α-L-arabinofuranosidase, *EC* 3.2.1.55) were also observed.

Forage contains various carbohydrate polymers which are the main source of energy for herbivores. The digestion of cellulose and fibrous plant material depends on the ability of endosymbiotic micro-organisms to break down the walls of plant cells and to ferment the carbohydrates. Like the population of the rumen, protozoa and bacteria in the caecum colonize the plant fragments to which they are attached (Bonhomme-Florentin, 1985).

A network of enzymes is needed to hydrolyse these substrates. A study has been made of the distribution of pectinolytic enzymes (polygalacturonase, *EC* 3.2.1.15; pectin lyase, *EC* 4.2.2.10; pectinesterase, *EC* 3.1.1.11) and hemicellulase enzymes (xylan endo-1,3-β-xylosidase, *EC* 3.2.1.32; β-mannosidase, *EC* 3.2.1.25), in three fractions of the caecum contents: acellular fluid (AF), bacteria (B), and protozoa plus bacteria (PB).

MATERIALS AND METHODS

Samples of caecum contents were collected from six slaughtered horses. Each sample was used to prepare three fractions (AF, B and PB) by the procedure summarized in Fig. 1. The fractions were then examined for enzyme activity as follows.

Incubations

Polygalacturonase. Two incubation media were used. One consisted of 0·25 ml polygalacturonic acid solution (10 g/l; pH adjusted to the desired value with 0·5 M-sodium hydroxide), 0·5 ml 20 mM-*N*-tris-(hydroxymethyl) methyl-2-aminoethanesulphonic acid (TES; pH 6·0, 7·0 or 8·0), and 0·25 ml enzymic extract (PB, B or AF). Two controls were used: (1) 0·25 ml polygalacturonic acid solution (10 g/l) with 0·75 ml TES buffer, (2) 0·25 ml enzymic extract with 0·75 ml TES. The other medium consisted of 1 ml enzymic extract added to a pectin solution (10 g/l) in a phosphate–citrate buffer (pH 7·0, 15 ml) (MacIlvaine, 1921). Incubation was carried out at 40°.

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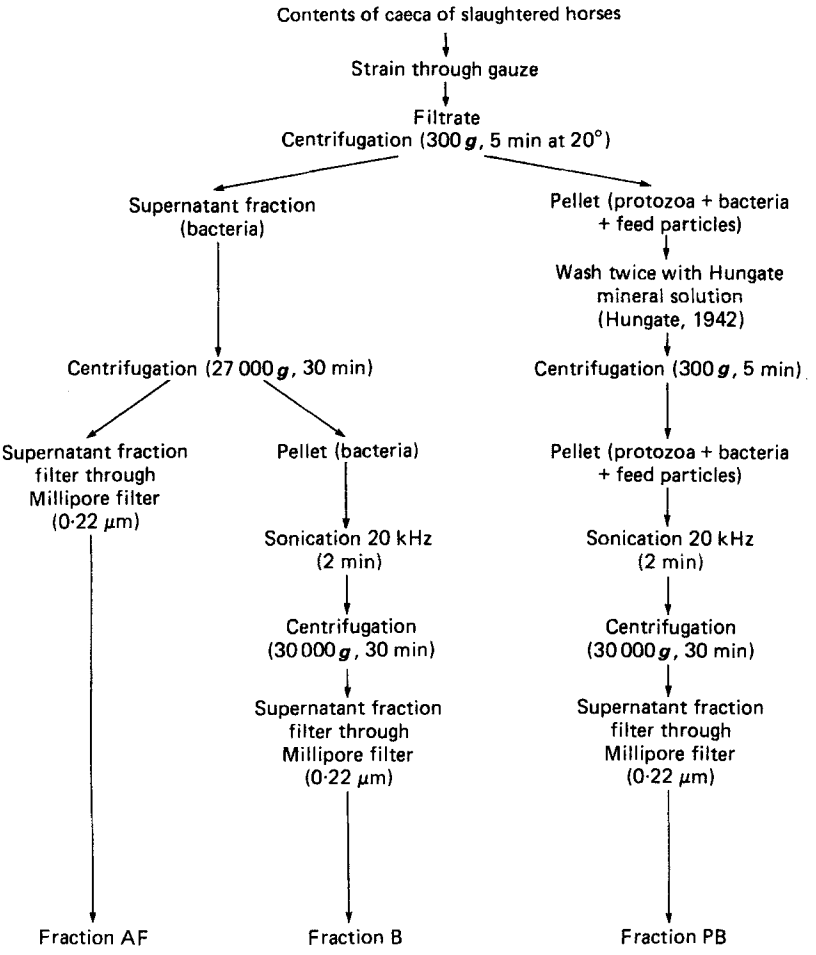

Fig. 1. Fractionation of horse caecum contents into acellular fluid (AF), bacteria (B) and protozoa plus bacteria (PB).

Pectin lyase. A pectin solution (20 g/l) in distilled water was centrifuged at 34000 *g* for 1 h to separate out the colloidal particles. Phosphate–citrate buffer solutions (3 vol.) of various pH values ranging from 4·0 to 8·0 were added to the supernatant fractions (1 vol.). This substrate solution (2 ml) and the enzymic extract (0·1 ml diluted 1:3, v/v) were incubated at 40°.

Pectinesterase. The incubation medium consisted of 1 ml of the substrate (a pectin solution (20 g/l) in phosphate–citrate buffers with pH values of 5·0, 6·0 and 7·0) and 0·25 ml enzymic extract. Incubation was carried out at 40° for 18 h.

Hemicellulases. The incubation medium comprised 0·2 ml hemicellulose solution (10 g/l) and 0·5 ml enzymic extract buffered with 0·5 ml citrate buffer solution (0·1 M), pH 5·0 and 6·0, or TES buffer (20 mM), pH 7·0 and 8·0. The hemicelluloses used were obtained from Sigma Chemical Co., Poole, Dorset. They were xylan (from oat spelts) dissolved in 0·5 M-sodium hydroxide and adjusted to the desired pH value with 1 M-hydrochloric acid, mannan (from *Saccharomyces cerevisiae*), arabinogalactan (from larchwood) or galactomannan (guar gum). Incubation was carried out at 40° for 0·5 and 5 h.

Glycosidases. The incubation media consisted of 0·2 ml *p*-nitrophenyl derivative, β-D-xylopyranoside (3 mg/ml water), β-D-mannopyranoside (3 mg/4 ml water) or α-L-arabinofuranoside (3 mg/4 ml water), together with 0·7 ml 200 mM-phosphate buffer, pH 7·0, or 100 mM-citrate buffer, pH 5·0 and 6·0, and 0·1 ml enzymic extract (diluted 1:3, v/v). Incubation was carried out at 40° for 2 h. In all incubations, controls were included, where either the substrate or the enzymic extract were omitted.

Analytical procedures

Viscosity. The variation in viscosity of the pectin (10 g/l) solution in the second incubation medium (see p. 185) was measured by means of an Ostwald viscometer. The intrinsic viscosity (η_i) was estimated with the Hess & Philipoff (1937) formula:

$$\eta_i = 8(\eta_r^{\frac{1}{8}} - 1)/C_s,$$

where η_r is the relative viscosity and C_s is the concentration of substrate (g/l).

Reducing sugar. The reducing sugar produced was measured according to the Nelson (1944) microcuprimetric method as galacturonic acid for determining polygalacturonase activity, and as xylose, mannose and galactose for determining hemicellulase activity.

Measure of absorbance. For determining pectin lyase activity, the absorbance was measured at 235 nm at time zero (t_0) and after various periods of incubation. One unit of activity is defined as an increase in optical density of 0·1 at 235 nm in 2 h expressed per ml enzymic extract. Specific activity is expressed on a per mg protein basis.

Gas–liquid chromatography and thin-layer chromatography. The pectinesterase activity was determined by gas–liquid chromatography as methanol produced by the breakdown of pectin in a 20 g/l solution. After the incubation period, butan-2-ol (0·3 μl) was added to the incubation medium as an internal standard. Then, ammonium sulphate (3·6 M) and sodium dihydrogen phosphate (1 M) were added, and the mixture was left overnight at 4°. The methanol released was measured in a gas–liquid chromatograph (Intersmat IGC 121 FL) with a stainless-steel column (2 m in length, 3·18 mm in diameter) and a Porapak Q phase (100–120 mesh). The settings were as follows: oven 180°, injector 200°, detector 220°. The carrier gas was nitrogen at a pressure of 1 bar.

For determining polygalacturonase activity, after 6 h incubation, the breakdown of polygalacturonic acid was determined by ascending chromatography on silica gel (F1500, Merck). Two solvents were used: (1) acetic acid–pyridine–ethyl acetate–water (1:5:5:3, by vol.), and (2) butanol–acetic acid–water (4:3:3, by vol.). Ammoniacal silver nitrate (Trevelyan *et al.* 1950) and *p*-anisidine were used as localizing reagents.

For determining hemicellulase activity, after 6 h incubation, the breakdown of xylan or mannan was evaluated by ascending chromatography on silica gel (F1500). There were two runs in the solvent (butanol–pyridine–water 6:4:3, by vol.). The localizing reagents used were *p*-anisidine for mannose and aniline phtalate for xylose.

Colorimetric methods. For determining glycosidase activities with nitrophenyl derivatives as substrates, the released *p*-nitrophenol was determined (after the reaction had been stopped by the addition of 2 ml 0·2 M-sodium borate buffer, pH 9·8) from a standard range of *p*-nitrophenol in buffer solution from 10 to 100 μM. The absorbance was measured at 400 nm. The results are expressed as μmol *p*-nitrophenol/h per mg protein. Protein in each fraction (PB, B and AF) was determined according to the method of Lowry *et al.* (1951).

RESULTS

Samples from different horses showed variations in flora and fauna. Results given in the tables and described in the following sections are for single samples, but although absolute

Table 1. *Polygalacturonase* (EC *3.2.1.15*) *activity of fractions of horse caecum contents*

Fraction* incubated with polygalacturonic acid	nmol Galacturonic acid/min per mg protein		
	pH 6·0	pH 7·0	pH 8·0
AF	21	26	30·7
B	21	18·3	27·5
PB†	157	237·6	256

AF, acellular fluid; B, bacteria; PB, protozoa plus bacteria.
* Protein values of fractions (μg/ml incubation medium using bovine albumin as standard): AF 480, B 240, PB 275; for details of fractions, see Fig. 1.
† *Cycloposthium*, 9×10^4/ml.

Table 2. *Changes in the intrinsic viscosity* (η_i) *of a pectin solution* (*10 g/l*) *incubated with fractions of horse caecum contents**

Incubation period (min)	η_i					
	PB_1†	PB_2†	B_1	B_2	AF_1	AF_2
0·5	0·269	0·272	0·243	0·259	0·237	0·233
5	0·263	0·262	0·240	0·253	0·233	0·229
10	0·253	0·253	0·237	0·251	0·231	0·227
15	0·248	0·225	0·235	0·245	0·225	—
30	0·242	0·167	0·225	0·240	0·222	0·226
40	0·238	0·146	0·225	0·238	0·218	0·226

AF_1, AF_2, acellular fluid; B_1, B_2, bacteria; PB_1 PB_2, protozoa plus bacteria.
* Protein values of the fractions (μg/ml incubation medium using bovine albumin as standard) PB_1 195, PB_2 375, B_1 390, B_2 280, AF_1 260, AF_2 250.
† Ciliate composition of PB fractions: PB_1 *Blepharocorys* $1{\cdot}6 \times 10^6$/ml; *Cycloposthium* $6{\cdot}2 \times 10^4$/ml; PB_2 *Cycloposthium* $3{\cdot}4 \times 10^5$/ml; *Didesmis*, *Blepharocorys*, *Paraisotricha*.

values varied between samples, the patterns of relations between the fractions PB, B and AF obtained from different caecum contents were consistent.

Pectinolytic activity of the fractions

Three enzymic pathways for degradation of pectin were observed. Two were depolymerase activities, the first causing hydrolysis of $1 \rightarrow 4$ glycosidic linkages (polygalacturonase) and the second resulting in the β elimination from the esterified carboxy group to give unsaturated uronides (pectin lyase). The third is a saponifying enzymic activity which hydrolyses the methoxyl groups from the pectin (pectinesterase). The results of the breakdown of polygalacturonic acid to galacturonic acid are shown in Tables 1 and 2. This activity was found mainly in the PB fraction, as indicated by the amount of reducing sugars determined as galacturonic acid. The activities per mg protein in fractions B and AF were comparable, but the latter contained twice as much protein. In the four different samples of caecum contents examined, the decrease in the intrinsic viscosity of a pectin solution (10 g/l) was two to six times more in fraction PB than in fraction B. The decrease was four times as great when the PB fraction was composed mainly of *Cycloposthium* spp. (PB_2) than

Table 3. *Chromatographic results of the degradation of polygalacturonic acid by the fractions of horse caecum contents*

Solvent*...	1		2		
Fraction	R_f	R_G	R_f	R_G	Identification
AF+PG	0·23	0·25	—	—	TG
	0·68	0·76	—	—	—
B+PG	0·23	0·25	0·22	0·44	TG
			0·34	0·67	DG
			0·49	0·95	MG
			0·62	1·2	Galactose
PB+PG	0·23	0·25	0·22	0·44	TG
	0·50	0·56	0·34	0·67	DG
	0·77	0·85	0·5	0·97	MG
			0·58	1·12	Galactose
MG	0·9	1	0·51	1	—
Galactose	—	—	0·56	1·1	—

$R_f = X/S$; $R_G = X/G$, where the distance travelled by the substance (TG, DG, MG, galactose) is X, by the galacturonic acid is G and by the solvent front is S.

PG, polygalacturonic acid; MG, galacturonic acid; DG, digalacturonic acid; TG, trigalacturonic acid.

* Solvent 1, acetic acid–pyridine–ethyl acetate–water (1:5:5:3, by vol.), location reagent ammoniacal silver nitrate; solvent 2, butanol–acetic acid–water (4:3:3, by vol.), location reagent *p*-anisidine.

Table 4. *Specific pectin lyase* (EC *4.2.2.10*) *activity* of fractions of horse caecum contents*†

	Incubation medium		
	AF+pectin	B+pectin	PB+pectin
Incubation medium pH (incubation period 2 h)			
5·0	1·3	2·7	0·6
6·0	tr	2·6	6
7·0	2	7·8	24·5
8·0	tr	0·5	9
Incubation period (h) (pH 7)			
2	2	7·8	24·5
4	tr	11·2	24·5
21	tr	20·2	26·3

tr, trace amounts; AF, acellular fluid; B, bacteria; PB, protozoa plus bacteria.

* One unit of activity is defined as the increase in optical density of 0·1 at 235 nm in 2 h expressed per ml enzymic extract, specific activity is expressed in units per mg protein.

† Protein values of the fractions (mg/ml incubation medium using bovine albumin as standard): AF 1·4, B 2, PB 1; for details of fractions, see Fig. 1.

when it also contained large numbers of *Blepharocorys* spp. (PB_1) (Table 2). These results are put forward as an example of viscosity variations induced by fractions from different caecum contents. They show that pectinolytic activity is dependent on the qualitative composition of caecum microfauna. Chromatographic analysis indicated that polygalacturonic acid was broken down into tri-, di- and monogalacturonic acids by fractions

Table 5. *Pectinesterase* (EC *3.1.1.11*) *activity of fractions of horse caecum contents** (*nmol methanol/h*)

pH of incubation medium...	5·0	6·0	7·0
AF + pectin	7·8	8·1	7·5
B + pectin	60·3	50·6	20·3
PB + pectin	41·5	317·2	23·4

AF, acellular fluid; B, bacteria; PB, protozoa plus bacteria.

* Protein values of fractions (mg/ml incubation medium using bovine albumin as standard): AF 0·8, B 0·9, PB 1·2.

Table 6. *Hemicellulase activity of fractions of horse caecum contents**

Fraction	Substrate	nmol sugar produced/h per mg protein			
		pH 5·0	pH 6·0	pH 7·0	pH 8·0
AF	X	1190	1445	1710	511
B		1314	1134	540	318
PB		2210	2100	1300	900
AF	M	390	462	354	390
B		120	tr	tr	tr
PB		tr	tr	540	540
AF	GM	49	21	tr	tr
B		90·7	63	31·6	49·4
PB		311·5	295	177·6	122
AF	AG	84	84	21	21
B		55·5	51·3	tr	tr
PB		110	104	54·6	68·3

tr, trace amounts; AF, acellular fluid; B, bacteria; PB, protozoa plus bacteria; X, xylan; M, mannan; GM, galactomannan; AG, arabinogalactan.

* Protein values of the fractions (mg/ml of incubation medium using bovine albumin as standard): AF, 0·6, B 1·2, PB 2 and 1·6 (with GM and AG respectively); for details of fractions, see Fig. 1.

Table 7. *Glycosidase activity of fractions of horse caecum contents* on two* p*-nitrophenyl derivatives* (*μmol* p*-nitrophenol/h per mg protein*)

pH of incubation medium...	5·0	6·0	7·0	8·0
B + NX	26·7	6·7	tr	tr
PB + NX	177·5	1100	537·5	tr
B + NA	23	227	143	60
PB–NA	135	792·5	647·5	280

tr, trace amounts; B, bacteria; PB, protozoa plus bacteria; NX, *p*-nitrophenyl β-D-xylopyranoside; NA, *p*-nitrophenyl α-L-arabinofuranoside.

* Protein values of the fractions (μg/ml of incubation medium using bovine albumin as standard): B 150, PB 200; for details of fractions, see Fig. 1.

PB and B. With fraction AF, only a small amount of trigalacturonic acid was detected (Table 3).

The depolymerizing activity (pectin lyase) was concentrated in fractions B and PB (Table 4). Pectin lyase activity of fraction B was particularly evident after 20 h of incubation. The PB fraction reached its peak of activity after 2 h of incubation. The optimum pH was 7·0. The AF fraction had no pectin lyase activity. The de-esterification of pectin (by pectinesterase), with the production of free carboxyl groups and methanol was greatest in fraction PB. At the optimum pH of 6·0, the pectinesterase activity of fraction PB was higher than that of fraction B. Fraction AF had very weak activity (Table 5).

Hemicellulase activity of the fractions.

As regards the various hemicellulase activities, all three fractions contained significant xylan endo-1,3-β-xylosidase activity. Chromatrographic analysis indicated the breakdown of xylan into xylose. With fraction B, no chromatographic spots were observed which could be xylodextrins, xylotriose or xylobiose. Galactomannan was only partially broken down into mannose and galactose by all three fractions in a pH range between 5·0 and 6·0; fraction PB had the highest activity. Arabinogalactan was broken down into arabinose and galactose by all three fractions in the same pH zone. Fraction PB contained twice the activity of fraction B (Table 6). As regards glycosidase activities, xylan 1,4-β-xylosidase and α-L-arabinofuranosidase were observed in fractions PB and B on the nitrophenyl derivatives β-D-xylopyranoside and α-L-arabinofuranoside (Table 7). The optimum pH of these activities was 6·0 for fraction PB. Fraction AF showed no activity. The *p*-nitrophenyl β-mannopyranoside was partially broken down. Fractions PB and B released 10 μmol *p*-nitrophenol/h per mg protein at pH 5·0.

DISCUSSION

According to Hintz (1969), horses digest fibre only two-thirds as efficiently as ruminants do. Observing faecal particles under the electron microscope, Grenet *et al.* (1984) also reported that horses digest plant cell walls less well. Harbers *et al.* (1981), who studied the digestion of three types of forage by horses, found that the hemicelluloses of hay from *Andropogon gerardi*, *Festuca arundinacea* and *Bromus inermis* have apparent digestibility coefficients of 0·542, 0·577 and 0·668 respectively. Peltonen *et al.* (1978) observed that the microbial population and its activity in the digestive tract are particularly influenced by the physico-chemical composition of the forage. As in the rumen, the endosymbiotic micro-population of the caecum and colon decomposes cell-wall carbohydrates (hemicellulose and pectin). These polysaccharides are broken down through an interaction of depolymerases and glycosidases; these latter isolate the side chains of the main chain and hydrolyse the fragments of oligosaccharides released by the activity of the depolymerases. Pectin, the polymer of galacturonic acid which represents a small proportion of the cell-wall carbohydrates in forage, is broken down in the rumen by most of the ciliates (*Isotricha*, *Spirotricha*, *Ophryoscolex*, *Epidinium* and *Polyplastron* spp.) (Abou Akkada & Howard, 1961; Abou Akkada *et al.* 1963; Wright, 1960, 1961; Mah & Hungate, 1965), with the release of oligouronides, galacturonic acid and methanol. Similarly in the caecum, the ciliates, mainly *Cycloposthium* spp. but also *Blepharocorys* spp., actively degrade pectic substances by means of three enzymes: polygalacturonase, pectin lyase and pectinesterase. The bacterial fraction is linked to this activity in the same way as in the rumen, where there is synergy between bacteria hydrolysing the pectic substances and those using the end-products of this hydrolysis (Prins, 1977). The hemicelluloses in forage, which represent 30–40% of the polysaccharide complex, are mainly β-1,4-xylans. In ruminants, bacteria

(Dehority, 1965, 1973) and entodiniomorph ciliates (*Ophryoscolex*, *Epidinium* and *Polyplastron*) (Bailey *et al.* 1962; Abou Akkada *et al.* 1963; Abou Akkada & El Shazly, 1965; Clarke, 1977) participate in the hydrolysis of xylan, arabinan and mannan. The distribution of enzymes which degrade cell-wall polysaccharides in the caecum, shows that the protozoal fraction degrades cellulose (Bonhomme-Florentin, 1974) and hemicelluloses, and has glycosidases linked to the depolymerization of these structural constituents. The cellulose is broken down by one cellulase (*EC* 3.2.1.4) and one β-D-glucosidase (*EC* 3.2.1.21) into glucose. The hemicelluloses are hydrolysed into xylose, arabinose, and galactose by glycanhydrolases (xylan endo-1,3-β-xylosidase, *EC* 3.2.1.32; α-L-arabinanase), by glycosidases (xylan 1,4-β-xylosidase, *EC* 3.2.1.37; α-L-arabinofuranosidase, *EC* 3.2.1.55), as shown previously, and by β-galactosidase (*EC* 3.2.1.23) (Bonhomme-Florentin, 1986*a*). The β-mannosidase (*EC* 3.2.1.25) activity was weak.

The bacterial fraction also participates mainly in the form of xylanase activity. The contents of the caecum are heterogeneous, as are those of the rumen. They include a complex solid mass of digesta and a liquid phase and therefore offer various micro-environments. Observations using the electron microscope (Bonhomme-Florentin, 1985, 1986*b*) show micro-organisms attached to the plant-cell constituents. In the present study, the hydrolytic activities correspond to those of the micro-organisms in the caecal liquid phase and, probably, as in the rumen (Williams & Strachan, 1984), the attached micro-organisms are even more important in the breakdown of structural polysaccharides. The actual enzyme activities in the caecum are probably higher than the activities observed in the present work.

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