Prediction of the Digestibility of Forages by Treatment of their Cell Walls with Cellulolytic Enzymes

Roy D. Hartley, Edwin C. Jones and John S. Fenlon

Grassland Research Institute, Hurley, Maidenhead, Berks. SL6 5LR, England
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A method is described for the prediction of the in vivo digestibility (d.m.d.) and in vitro cell wall digestibility of grasses: it involves incubating grass cell walls with a commercial cellulase for 16 h and measuring the optical density of the filtrate at 324 nm (o.D.). The prediction equation is based on a highly significant correlation \( r = 0.978 \) between o.d. and d.m.d. using a total of 27 samples of three species of grass with d.m.d. between 60 and 83. The method could not be used for the prediction of d.m.d. of red clover and sainfoin due to their filtrates having low o.D. values. A second method for the prediction of d.m.d. of grasses is also described and is based on a highly significant correlation between d.m.d. and the percentage of cell walls digested by cellulase \( r = 0.962 \): similar comparisons for the legumes gave lower correlation coefficients. Using either the o.D. or gravimetric method, inclusion of cell wall content in the regression equations reduced the s.d. of d.m.d. predictions.

1. Introduction

Several laboratory methods have been proposed for the estimation of the digestibility of forages for ruminants. Various chemical methods have been suggested but are not entirely satisfactory as several cell-wall components need to be determined before sufficiently precise estimates of digestibility can be obtained.\(^1\)\(^2\) Several workers\(^3\)-\(^5\) have shown lignin contents to be of little value in predicting forage digestibility. The rumen liquor-acid pepsin in vitro method of Tilley and Terry\(^6\) is employed in many forage-evaluation laboratories, but this method suffers from the necessity of keeping fistulated animals as the source of rumen liquor and from difficulties in reproducibility due to variations in enzymic activity of the liquor. In attempts to overcome these difficulties, commercial cellulase preparations have been proposed.\(^7\)\(^8\)

In the work reported here we have isolated cell walls before treating them with a commercial cellulase, for two reasons. First, the accuracy of predicting digestibility is likely to be increased if possible interference with enzyme activity by soluble contents of the cells is prevented and, second, cell-wall content can be a useful parameter for studies in animal nutrition, for example for prediction of the voluntary intake of forages.\(^9\) Digestibility was estimated by optical density measurements of filtrates obtained by treatment of cell walls with cellulase and by weighing residues.

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2. Experimental

2.1. Source of cellulase
The cellulase, which was of fungal origin (Oxyporus sp., a Basidiomycete), was supplied by E. Merck, Darmstadt, Germany, cat. no. 24525; activity 90 mU/mg. The assay conditions and definition of the unit of activity are obtainable from E. Merck.

Several commercial cellulases were examined and E. Merck cellulase was chosen because of its greater capacity for digesting grass cell walls.

2.2. Plant material
Italian ryegrass (Lolium multiflorum, cv. Tetila Tetrone), perennial ryegrass (Lolium perenne, cv. Endura), timothy (Phleum pratense, cv. S 352), red clover (Trifolium pratense, cv. Hungaropoly) and sainfoin (Onobrychis viciefolia, cv. Cotswold Common) were harvested in 1969 on 9 successive occasions during primary growth to provide samples before and after inflorescence as described by Terry, Osbourn and Fenlon,2 freeze-dried and ground to pass a 0.8-mm sieve. Ash-free lignin (L) was determined by the method of Van Soest and Wine.10

2.3. Separation of cell walls
Cell walls were prepared from the dry plant material (1.0 g) by the method of Van Soest and Wine11 except that Na₂SO₄ and decahydronaphthalene were omitted. The cell walls were obtained by filtration (No. 1 porosity sintered glass crucible) and washed successively with hot water (60 ml), acetone (60 ml) and ether (60 ml), dried over silica gel and weighed.

2.4. Digestion of cell walls with cellulase
Cellulase was dissolved in buffer (0.2 M-NaOH-acetic acid, pH 4.8, containing 0.02% NaN₃), filtered (No. 1 crucible), washed with buffer and the filtrate plus washings made up with buffer to a concentration of 2.5 mg cellulase/2.0 ml buffer. Cell walls (30 mg) were incubated in the dark at 37 °C for 16 h with 2.0 ml of the cellulase solution. As a control, each sample was incubated by the same method but without the addition of cellulase. The mixtures were filtered (No. 1 crucible) in “white” fluorescent light in the absence of daylight and the residues washed with water. The residues were dried over silica gel and weighed. The weights of residues left after cellulase treatment were corrected by subtracting the weight lost by treatment with buffer; this correction was <2% of cell walls. The filtrate plus washings were made up to 25 ml and shaken.

2.5. Examination of filtrates by ultraviolet (u.v.) absorption
Filtrates were scanned from 250 to 370 nm and optical density measurements recorded at the absorption maxima and corresponding wavelengths (i.e. \( \lambda_{\text{max}} \)). Solutions were stored, when necessary, at 4 °C in the dark and were manipulated in fluorescent light as above. No change in optical density occurred over a 3-day period.

NaOH (10 N, 1.1 ml) was added, in fluorescent light, to an aliquot of each filtrate (10 ml) and the mixture shaken and allowed to stand 45 min before determining optical density as above. These solutions were stable for at least 3 h if kept in the dark.
2.6. Determinations of digestibility of plant materials by the in vivo method and by the rumen liquor–acid pepsin in vitro method

The coefficients of apparent digestibility of the forage dry matter (d.m.d.) and of their cell walls (c.w.d.) were determined from the measured intake of feed and output of faeces using groups of 3 to 5 mature sheep.\(^2\)

The apparent digestibility of the forages was also determined by the rumen liquor–acid pepsin in vitro method.\(^2,6\)

3. Results and discussion

3.1. Grasses

Perennial rye grass was chosen for this study as it is commonly used for ruminant feeding, together with Italian ryegrass and timothy as these two species differ considerably in their botanical and biochemical characteristics.\(^2\) A series of harvests during primary growth of each of these three species was obtained to allow investigation of grass before and after inflorescence.

In our earlier work\(^1,2,13\) highly significant correlations were found between the ultraviolet (u.v.) absorption of the untreated filtrate at \(\lambda_{\text{max}}\ 290\) or \(324\) nm and the percentage of a cell-wall fraction digested by cellulolytic enzymes during 16 h at \(37\) °C. This cell-wall fraction was obtained from glasshouse-grown grass. The u.v.-absorption was due to the presence of carbohydrate esters of phenolic acids in the filtrate. A typical u.v.-spectrum of a filtrate from one of the field-grown grasses used in the present work is shown in Figure 1 together with the corresponding spectrum after treatment with NaOH to release the sodium salts of the phenolic acids.

U.v.-absorption measurements on the untreated filtrates at \(\lambda_{\text{max}}\ 324\) nm and the digestibility of cell walls of grasses using a commercial cellulase are shown in Table 1 together with d.m.d. and lignin contents. The optical density at \(\lambda_{\text{max}}\ 324\) nm (o.D.) of the untreated filtrate from cellulase treatment of cell walls, was the only measurement required to predict in vivo cell-wall digestibility (c.w.d.) or d.m.d. of the grasses. For each grass, the correlation coefficients \((r)\) obtained by comparison of c.w.d. or d.m.d. with o.D. were greater than 0.98. Although the regression equations for the prediction of c.w.d. or d.m.d. were significantly different for the three species of grass, the following equations for pooled grasses allowed prediction with low standard deviation (s.d.):

\[
\text{c.w.d.} = 39.84 + 48.91\ \text{o.D.} \quad r = 0.979\ (P < 0.01),\ \text{s.d.} = 2.19
\]

and

\[
\text{d.m.d.} = 48.30 + 35.57\ \text{o.D.} \quad r = 0.978\ (P < 0.01),\ \text{s.d.} = 1.65
\]

Similarly, accurate predictions of c.w.d. or d.m.d. were obtained from optical densities of the filtrates at \(\lambda_{\text{max}}\ 290\) nm before NaOH treatment or at \(\lambda_{\text{max}}\ 296\) or \(338\) nm after treatment with NaOH. Measurement at \(\lambda_{\text{max}}\ 324\) nm was preferred without NaOH treatment as absorption due to cellulase was negligible at this wavelength and, for simplicity, the extra step of NaOH addition was avoided.

Analogous regression equations were obtained for the prediction of c.w.d. and d.m.d. from the pooled results of the grasses from the percentage of cell walls digested by cellulase (c.w.d.,):
c.w.d. = 45.91 + 0.686 c.w.d.  \hspace{1cm} r = 0.959 (P < 0.01), s.d. = 3.06 \hspace{1cm} (3)

and

d.m.d. = 52.63 + 0.501 c.w.d.  \hspace{1cm} r = 0.962 (P < 0.01), s.d. = 2.14. \hspace{1cm} (4)

Low s.d. values for equations (1) and (3) might be expected from earlier work.\textsuperscript{12}

On the other hand, the s.d. values obtained with equations (2) and (4) were better than might be expected as no allowance was made for the proportion of cell contents to cell walls. In order to consider the digestibility of cell contents and cell walls separately, Van Soest and Jones\textsuperscript{1} proposed the following equation for prediction of d.m.d.:

\[
d.m.d. = 0.98(100 - \text{n.d.f.}) - 12.9 + \text{n.d.f.} \left(1.473 - 0.789 \log \frac{100L}{\text{a.d.f.}}\right) - 3.0(\text{SiO}_2) \hspace{1cm} (5)
\]

where n.d.f. = neutral detergent fibre,\textsuperscript{a} L = ash-free lignin, a.d.f. = acid detergent fibre and SiO\textsubscript{2} = silica, all determined as \% of dry matter. The first factor in equation (5) estimates the digestibility of cell contents, the second factor (=12.9) allows for bacterial cell wall plus endogenous excretion from the animal, the third factor estimates the digestibility of cell walls and the last factor is a correction for the presence of silica.

Osbourn \textit{et al.}\textsuperscript{14} have recently obtained the following equation using 54 forage samples, including the grass samples examined in the current work:

\textit{This fraction is similar to cell walls (c.w.) isolated in the present work.}
TABLE 1. Data from cellulase treatment of cell walls of grasses in relation to digestibility and lignin content

<table>
<thead>
<tr>
<th>Sample</th>
<th>Date of harvest</th>
<th>Cell walls (c.w., % of dry matter)</th>
<th>Cell walls digested by cellulase (c.w.d., %)</th>
<th>Optical density of filtrate from treatment of cell walls with cellulase (O.D.)</th>
<th>In vivo cell-wall digestibility (c.w.d.)</th>
<th>Dry matter digestibility</th>
<th>In vivo method (d.m.d.)</th>
<th>In vitro method (d.m.d.RL)</th>
<th>Lignin (L, % of dry matter)</th>
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<td>29/4</td>
<td>30.9</td>
<td>66.7</td>
<td>0.94</td>
<td>85.8</td>
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<td>82.3</td>
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* Filtrate diluted to 25 ml for measurement of optical density at λ_{max} 324 nm using 1 cm cell.
As an alternative method for the prediction of d.m.d. using cellulolytic methods, we have used the following equation based on equation (6):

\[
d.m.d. = 0.96(100 - c.w.) - 11.1 + c.w. \left( \frac{c.w.d.}{100} \right)
\]

where c.w. = cell walls (% of dry matter). C.w.d. was determined either from o.d. using equation (1) or from c.w.d.\(_e\) using equation (3), giving the following equations:

\[
d.m.d. = 0.96(100 - c.w.) - 11.1 + \frac{c.w.}{100} (39.84 + 48.91 \text{ o.D.})
\]

\[ r = 0.983 \ (P < 0.01), \text{ s.d.} = 1.42 \]

and

\[
d.m.d. = 0.96(100 - c.w.) - 11.1 + \frac{c.w.}{100} (45.91 + 0.686 \text{ c.w.d.} \_e)
\]

\[ r = 0.980 \ (P < 0.01), \text{ s.d.} = 1.55 \]

Hence for the prediction of d.m.d., the s.d. values obtained using equations (8) and (9) were lower than those obtained using equations (2) and (4).

The o.d. and c.w.d.\(_e\) methods described above have given better s.d. values for prediction of in vivo d.m.d. than the cellulase method of Jones and Hayward\(^8\) (s.d. = 2.5). This might be expected as Jones and Hayward did not remove cell contents which may cause interference with enzyme activity. The temperature at which samples are dried before analysis could have an important influence on digestibility with cellulase: in the present work the samples were freeze-dried while in the case of Jones and Hayward the drying conditions were not defined.

The following regression equations were obtained for pooled grasses in order that d.m.d. values estimated previously by the rumen liquor–acid pepsin in vitro method (d.m.d.\(_{RL}\)) may be compared with results from the cellulolytic method, for example in long-term agronomic trials:

\[
d.m.d.\_{RL} = 49.02 + 32.74 \text{ o.D.} \quad r = 0.963 \ (P < 0.01), \text{ s.d.} = 1.97
\]

and

\[
d.m.d.\_{RL} = 52.77 + 0.467 \text{ c.w.d.} \_e \quad r = 0.960 \ (P < 0.01), \text{ s.d.} = 2.06.
\]

O.d. or c.w.d.\(_e\) can also be used for the prediction of L as shown by the following regression equations for pooled grasses:

\[
L = 7.015 - 6.247 \text{ o.D.} \quad r = -0.956 \ (P < 0.01), \text{ s.d.} = 0.41
\]

and

\[
L = 6.287 - 0.089 \text{ c.w.d.} \_e \quad r = -0.944 \ (P < 0.01), \text{ s.d.} = 0.44.
\]

Preliminary investigations have shown no change in activity of a given batch of cellulase stored for several months at 4 °C nor has any difference in activity between
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batches been detected: the supplier states that the enzyme does not suffer considerable losses in activity for a period of one year if stored at 4 °C.

3.2. Legumes

A series of red clover and sainfoin samples were examined by the same cellulolytic methods. In contrast to grasses, the filtrates obtained by treatment of the legume cell walls with cellulase all had low optical density between 250 and 370 nm. The samples contained approx. 10% of grass due to machine-harvesting; when the grass was removed from one sample of sainfoin before cell-wall separation the optical density at 324 nm of the filtrate, after cellulase treatment, was zero.

Regression equations for the estimation of c.w.d., d.m.d. and L from c.w.d. of sainfoin were different from those of red clover or grasses. Correlation coefficients for sainfoin were lower than those found for grasses: the r for sainfoin for c.w.d. and c.w.d. was 0.908 and for d.m.d. and c.w.d. 0.953 (8 d.f.). Red clover gave much lower r values: the r for c.w.d. and c.w.d. was 0.841 and for d.m.d. and c.w.d. 0.817 (7 d.f.). These lower r values compared with grasses were not due to a smaller number of samples; for example, the r for d.m.d. and c.w.d. for timothy (8 d.f.) was 0.988. The lower r values might be due to the narrower range of values generally found for legumes compared with grasses. For example, c.w.d. for red clover varied between 26 and 42 while in vivo d.m.d. varied between 61 and 77.

4. Conclusions

For the estimation of d.m.d. of the three grasses, the u.v.-absorption at 324 nm of filtrates obtained by treatment of a known weight of cell walls with cellulase was the only measurement required. The method could not be used for red clover and sainfoin samples as the filtrates obtained by treatment of their cell walls with cellulase gave low absorption at 324 nm. This difference in spectral behaviour between grasses and legumes is being investigated. An alternative method for estimation of d.m.d. of grasses and legumes was to determine the percentage of cell walls digested by cellulase. Examination of a greater range of samples, including oven-dried forages, samples of low digestibility and regrowths, by the above methods is necessary to ascertain how widely the regression equations may be applied.

Acknowledgements

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References