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Hot alkali-labile linkages in the walls of the forage grass *Phalaris aquatica* and *Lolium perenne* and their relation to in vitro wall digestibility

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Dedicated to the memory of Profressor Jeffrey B. Harborne

Abstract

The factors affecting in vitro dry matter digestibility (IVDMD) of fully mature internodes of 150 lines of the forage grass, *Phalaris aquatica*, and internodes of 100 lines of perennial ryegrass (*Lolium perenne*), harvested just after anthesis, were investigated. The relationships between IVDMD and the contents of acetyl bromide lignin, and ester–ether linkages between lignin and wall polysaccharides, measured by hydroxycinnamic acids (HCAs) released by 4 M NaOH at 170 °C respectively, were determined. The regression analysis gave $r^2 = 0.05$ and 0.03 for the relation between IVDMD and lignin content and $r^2 = 0.51$ and 0.53 for the relation between IVDMD and the content of hot alkali-labile HCA (predominantly ferulic acid) for phalaris and ryegrass, respectively. These observations are interpreted in terms of the restricted accessibility of polysaccharide hydrolysing enzymes to their substrates in the forage cell walls by the covalent cross-linking of wall polymers through HCAs.

Keywords: *Phalaris aquatica*; *Lolium perenne*; Gramineae; Non-cellulosic polysaccharides; Grasses; Digestibility; Lignin; Ester–ether linkages

1. Introduction

The digestibility of cell walls in forage grasses by ruminants is a limiting factor in the utilization of wall polysaccharides as carbon and energy sources. Some walls such as those of parenchymatous cells are almost completely digested in the rumen, but others resist digestion. These are from vascular bundle, sclerenchyma and fibre cells, whose walls are lignified, and the outer walls of epidermal cells, whose walls are cutinised (Chesson et al., 1986; Hatfield, 1993; Wilson, 1993). This reduced digestibility has been attributed to the restricted accessibility of the polysaccharide hydrolases of the rumen microorganisms to cellulose and non-cellulosic polysaccharide substrates in the wall due to their physical encrustation by lignin or other non-carbohydrate wall components (see for example Van Soest, 1993).

In addition to physical associations of wall polysaccharides with one another and with lignin, cutin, suberin and proteins, covalent associations between these polymeric components in walls of grasses are also found. These are the quantitatively important covalent hydroxycinnamic acid (HCA) bridges between non-cellulosic polysaccharides, principally heteroxylans (Ishii, 1991; Lam et al., 1994a; Grabber et al., 1995, 1998, 2000) and the ester–ether bridges between the heteroxylans and lignin through hydroxycinnamic acids and dehydrodimers of hydroxycinnamic acids (di-HCAs) (Iiyama et al., 1990, 1994; Lam et al., 1992, 1994a,b). Other evidence points to the occurrence of bridges between esterified HCA on heteroxylans and wall proteins (Aeschbach et al., 1976; Saulnier et al., 1995; Oudgenog et al., 2002; Rhodes and Stone, 2002; Rhodes et al., 2002). There are additional possibilities for covalent associations between wall polymers (Bacic et al., 1988) through direct ether linkages between lignin and polysaccharides and

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direct ester linkages between lignin and uronic acids on noncellulosic polysaccharides. However there is no quantitative data on their content in walls of grasses.

Here we report relationships between in vitro dry matter digestibility (IVDMD) of mature intermodes of 150 lines of the forage grass, *Phalaris aquatica* harvested 14 weeks after anthesis, and 100 lines of perennial ryegrass (*Lolium perenne*) that were harvested just after anthesis, and their acetyl bromide lignin and ester–ether bridge contents, respectively.

2. Results and discussion

Lignin contents of internodes ranged from 15.9 to 31.3% oven dry material (ODM) and from 13.5 to 24.7% ODM, for phalaris and ryegrass respectively (Fig. 1). We measured the content of HCA ester–ether bridges based on our observation that in walls of wheat (*Triticum aestivum*) internodes (Lam et al., 1992, l994a) all ether-linked ferulic acid to lignin is also ester-linked to wall polysaccharides. No ether-only HCA was observed. Although ether linkages between lignin and HCAs are not cleaved by 1 M NaOH at room temperature overnight they are, however, substantially cleaved by 4 M NaOH at 170 °C (Lam et al., 1994b), because most of HCAs are linked at the benzyl position of lignin monomeric units (Lam et al., 2001). The content of HCA ester–ether bridges between wall polymers showed approximately normal distributions in both species (Figs. 2 and 3).

The normal distributions for lignin and HCA bridge contents among the lines of both plants (grown under identical environments in the same season) suggest that lignin biosynthesis and the formation of HCA bridges are both under multiple genetic control.

The relationships between IVDMD and acetyl bromide lignin content of phalaris and ryegrass internodes are shown in Figs. 4 and 5. Regression analysis gave $r^2 = 0.05$ and 0.03 for the relation between IVDMD and lignin content of phalaris and ryegrass, respectively. The relationships between IVDMD and the content of hot alicali-labile ferulic acid, that represents the content of ester–ether bridges between lignin and wall poly-

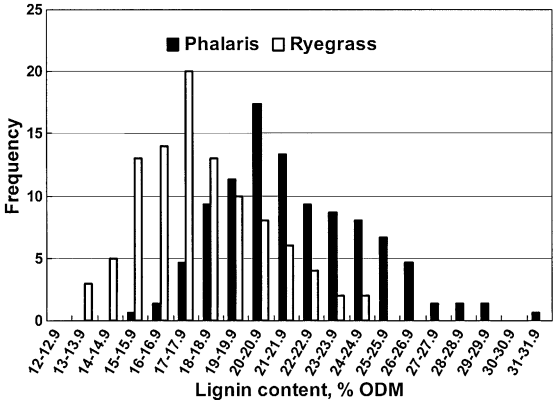

Fig. 1. Distribution of lignin contents in the walls of the forage grass *Phalaris aquatica* and perennial ryegrass (*Loliumperenne*) determined by an acetyl bromide procedure.

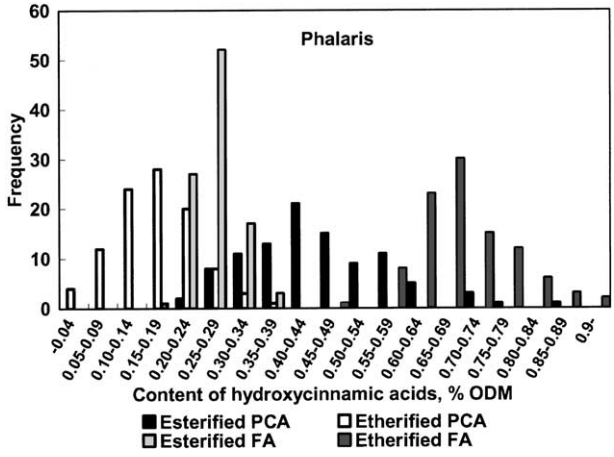

Fig. 2. Distribution of contents of hydroxycinnamic acids covalently bound to the wall polymers of the forage grass *Phalaris aquatica*.

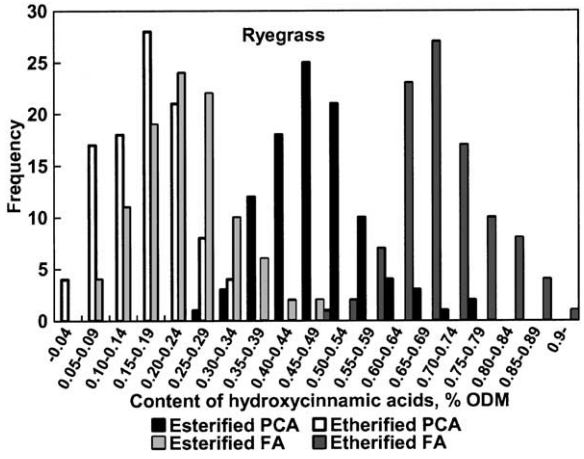

Fig. 3. Distribution of contents of hydroxycinnamic acids covalently bound to the wall polymers of perennial ryegrass (*Lolium perenne*).

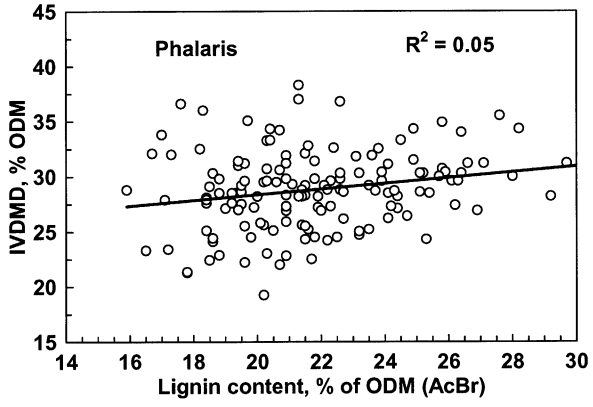

Fig. 4. Relationship between content of lignin and *in vitro*dry matter digestibility (IVDMD) of the forage grass *Phalaris aquatica* ($r^2 = 0.05$).

saccharides is shown in Figs. 6 and 7. Regression analysis gave $r^2 = 0.51$ and 0.53 for the relation between IVDMD and content of ether-linked ferulic acid in phalaris and ryegrass, respectively.

The present results show clearly that the ester–ether linked HCA content of mature phalaris and ryegrass internodes is negatively correlated with digestibility. These ester–ether bridges have now been well documented in walls of cells in various tissues from grasses and cereals (Scalbert et al., 1985; Sharma et al., 1986; Iiyama et al., 1990, 1994; Hatfield, 1993; Jacquet et al., 1995; Morrison et al., 1998; Hatfield et al., 1999; Casler and Jung, 1999; Grabber et al., 2000; Lapierre et al., 2001; Bunzel et al., 2001). The ester–ether bridges increase in concentration as grasses mature and the digestibility both in vitro (Lam et al., 1993) and in the rumen (Vailhé et al., 2000) decreases. The covalent bonding of heteroxylans to lignin through ferulic acid ester-linked to wall polysaccharides and ether-linked to lignin would restrict the accessibility of polysaccharide hydrolysing enzymes to their substrates in the internode particles. These covalent associations would tightly bond the wall polymers so that in the rumen swelling would be reduced and the approach of both polysaccharide hydrolases and HCA esterases (Borneman, 1990, 1992; Dalrymple, 1996; McSweeney et al., 1998) produced by rumen microorganisms to their potential substrates in wall fragments would be restricted. HCA esterases are able to cleave the esterified di-HCA in synthetic substrates (Garcia-Conesa et al., 1999) and diferulate cross-links are reported not to impede enzymatic polysaccharide hydrolase cleavage in unlignified maize walls (Grabber et al., 1998). However the situation in lignified walls would be quite different due to the covalent lignin–polysaccharide associations.

The lignin content of walls of forage grasses is widely held to be a major determinant of their digestibility in the rumen (see for example Van Soest, 1982; Vailhé et al., 2000) so that the absence of correlation between WDMD and acetyl bromide lignin content was unexpected and its interpretation is uncertain. The different methods used for digestibility assessment in the various studies may play a part. Although the WDMD digestibility procedure correlates with 'in rumen' methods (McLeod and Minson, 1978) the two environments are certainly not comparable with respect to particle size of the plant material, residence times and mode of presentation of enzymes to their substrates (soluble enzymes vs enzymes associated with surfaces of microorganisms).

Although lignin is of course involved in the ester–ether bridges, Casler and Jung (1999) have shown for smooth bromegrass (*Bromus inermis* Leyss) that the amount of ferulic acid etherified to lignin is not dependent on lignin concentration, and that the converse is also true. A related observation has been made in maturing wheat internodes where HCA content increased while lignin content was scarcely changing (Lam et al., 1993). Here the digestibilty decreases markedly during the period when HCA content was increasing. On the other hand Vailhé et al. (2000) found that in tall fescue (*Festuca arundinacea* Schreb.) both lignin content and the extent to which lignin was cova-

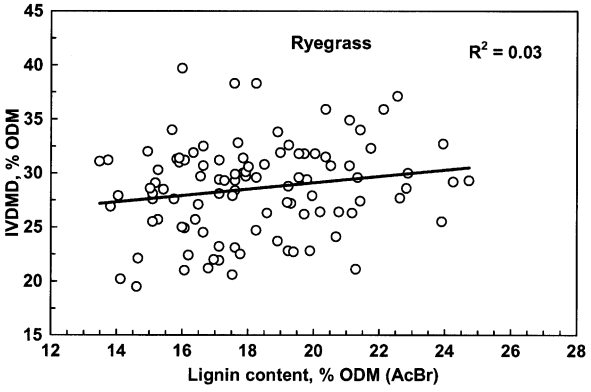

Fig. 5. Relationship between content of lignin and in vitro dry matter digestibility (IVDMD) of perennial ryegrass (*Lolium perenne*) ($r^2 = 0.03$).

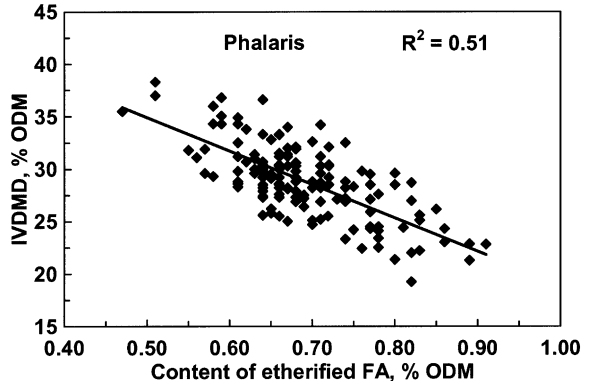

Fig. 6. Relationship between content of etherified ferulic acid (FA) and in vitro dry matter digestibility (IVDMD) of the forage grass *Phalaris aquatica* ($r^2 = -0.51$).

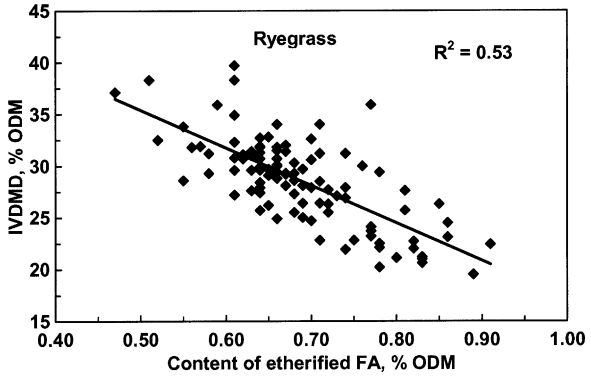

Fig. 7. Relationship between content of etherified ferulic acid (FA) and in vitro dry matter digestibility (IVDMD) of perennial ryegrass (*Lolium perenne*) ($r^2 = -0.53$).

lently bound to other wall polymers influenced the rate and extent of degradation.

The present observations support a conclusion that the lignin content of the walls of grasses alone is not in itself the most important regulator of IVDMD, but that the extent of covalent associations between wall polysaccharides and lignin is of major importance.

The natural variations in the content of HCA ester–ether bridges in phalaris and ryegrass, and their correlations with digestibility suggest that the content of hot alkali-cleavable ferulic acid will be a useful indicator of forage grass digestibility in conventional selection and marker-assisted breeding programs. The identification of the enzymes responsible for the formation of HCA ester linkage on heteroxylans of grasses would open the way for its selective down-regulation to generate grasses with low levels of HCA cross-linking (Lam et al., 1996).

There are many reports in the literature of improvements in the digestibility of forage and feed grasses following alkaline treatments (Van Soest, 1982; Morrison, 1991). The success of these procedures can be interpreted, at least in part (see Lapierre et al., 2001), in terms the alkaline disruption of ester-linked HCA making intermolecular cross-links between heteroxylans and ester–ether cross-links between heteroxylans and lignin and the consequent increases in the digestibility of the wall polysaccharides by hydrolytic enzymes.

3. Experimental

3.1. Plant material

Mature terminal (upper) internodes of the 150 lines of *Phalaris aquatica* L. plants supplied by Dr. Richard Culvenor, CSIRO Division of Plant Industry, Canberra, ACT and grown in the 1993 season were harvested 14 weeks after anthesis and freeze-dried. The terminal (upper) internodes of 100 lines of perennial ryegrass (*Lolium perenne* L.) were grown at the experimental farm of the School of Agriculture, University of Melbourne, Mount Derrimut, Victoria, just after anthesis in the 1993 season, immediately frozen and later freeze-dried. The dried samples were ground in a Wiley mill (Arthur H. Thomas, USA) to pass a 420-μm sieve and extracted with boiling 80% (v/v) aq. EtOH ×3 for 1 h each followed by extraction with H_2O at 40 °C overnight, under toluene, then dried at 40 °C in a vacuum oven prior to analysis (Lam et al., 1994b) (toluene was added to prevent micorbial growth).

3.2. Analytical methods

Lignin content of the extracted internodes was determined using the acetyl bromide procedure (Iiyama and Wallis, 1990) using 20.0 g^{-1} L cm^{-1} as the value of the specific absorption coefficient of lignin. The ester-linked and total covalently bound hydroxycinnamic acids in internode walls, previously extracted to remove unbound HCA, were released by treatments with 1 M NaOH overnight at room temperature and with 4 M NaOH for 2 h at 170 °C, respectively, were quantified using *m*-coumaric acid as an internal standard as described by Lam et al. (1994b). The content of etherified HCAs were calculated as the difference between the values of total and ester-linkage.

3.3. In vitro dry matter digestibility

The in vitro dry matter digestibility of the samples was determined by the procedure of McLeod and Minson (1978) using a porcine pepsin (Sigma Chemicals Co. Ltd. USA) in 0.125 M HC1 for 48 h at 37 °C, then a commercial broad spectrum, cell wall polysaccharide hydrolase preparation (Onozuka-SS, P 1500), from *Trichoderma viride*, Yakult Co. Ltd. Japan). The extracted sample was incubated in tubes for 48 h at 37 °C in 0.125 M HC1 containing 0.2% (w/v) pepsin (1:10 000) to remove the cell contents. After centrifugation, the residue was treated in 0.5 M acetic acid-sodium acetate buffer solution (pH 4.6) containing 2.5% (w/v) Onozuka polysaccharide hydrolase preparation for 48 h at 37 °C. The residue was washed twice with water before being dried at 105 °C and weighed.

All determinations were made in duplicate.

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