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Degradation of Perennial Ryegrass Leaf and Stem Cell Walls by the Anaerobic Fungus *Neocallimastix* sp. Strain CS3b

LOLKE SIJTSMA* AND BWEE TAN

Agrotechnological Research Institute (ATO-DLO), 6700 AA Wageningen, The Netherlands

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The degradation of cell walls isolated from stems and leaves of perennial ryegrass by the anaerobic fungus *Neocallimastix* sp. strain CS3b was studied in a defined medium. The combined cellulose and hemicellulose fraction represented 53.1 (wt/wt) and 63.3% (wt/wt) of the dry weight of control grass leaf and stem cell walls, respectively. In both leaf and stem cell walls, glucose was the major neutral monosaccharide, followed by xylose, arabinose, and galactose. After 2 days of fermentation with *Neocallimastix* sp. strain CS3b, treated cell walls contained smaller amounts of neutral sugars compared with those of undigested cell walls. These results were more evident for glucose, xylose, and arabinose than for galactose. Furthermore, the sugar content of leaf cell walls decreased before a decline in the sugar content of stem cell walls was observed. Data from formate and hydrogen production indicated that the growth of *Neocallimastix* sp. strain CS3b was completed in 4 days in the culture system used. During this period, the fungus liberated about 95% of the fermentable sugars in untreated material. On a percentage basis, no significant differences were found in final extent of degradation of glucose, xylose, and arabinose. Galactose, however, was degraded to a lesser extent.

Ruminants are able to digest high fibrous plant materials because of the presence of a complex microbial ecosystem in their digestive tracts. The microbes present produce the required enzymes and convert complex macromolecules into simple small molecules. Ruminant bacteria and protozoa have been held responsible for this conversion for over 100 years (16). In the mid-1970s, however, the presence of gut fungi was described (23); now it is acknowledged that these fungi are also involved in fiber digestion in herbivorous mammals.

Gut fungi are able to colonize fiber by anchoring plant materials by the rhizoid and produce a wide range of hydrolytic enzymes, including cellulases (3, 6, 19), hemicellulases (19, 22, 27), proteases (32), and feruloyl and *p*-coumaroyl esterases (7, 8). It is estimated that anaerobic fungi account for 8% of the biomass in the rumen (18), and it has been proposed (4) that they are initial colonizers of fibrous plant substrates in the rumen and that they make the fiber more accessible for secondary invasion by other rumen microorganisms (31). Furthermore, the extensive digestion of plant fiber by anaerobic fungi in vitro (24, 27, 29, 30) suggests that these organisms are well equipped to contribute to fiber degradation in vivo.

Anaerobic fungi are more prevalent in ruminants fed on stalky fibrous diets (5). This may be explained by the relatively long retention time (2 to 3 days) of large fibrous plant materials in the rumen (15), thus enabling more extensive colonization by anaerobic fungi. From feeding experiments with sheep, it appeared that upon treatment with antibiotics which removed the amount of fungi to undetectable levels, sheep ate 40% less of a straw-based diet than they ate with fungi present in the rumen (14).

Of the plant materials fed to ruminants, grass is one of the most important. Growth on and fermentation of glucose, cellulose, and several other sugars by anaerobic fungi have been well documented (20, 26, 29). A limited amount of information, however, is available concerning the degradation of individual cell wall sugars of grass cell walls by anaerobic fungi.

In this paper, we describe the degradation of cell wall sugars in stem and leaf cell walls of perennial ryegrass by the anaerobic fungus *Neocallimastix* sp. strain CS3b.

Strains and growth conditions. *Neocallimastix* sp. strain CS3b, isolated from rumen fluid of a sheep as described previously (27), was cultivated in the defined medium M2 (27, 28). Growth experiments were performed in triplicate in 20-ml penicillin flacons (Prins BV, Schipluiden, The Netherlands) with 15-ml aliquots of medium containing 100 mg of cell walls. Flacons were fitted with butyl rubber stoppers and aluminum caps, flushed with N_2-CO_2 (80:20), and autoclaved at 115°C. Prior to experiments, one transfer into a medium with cellobiose (0.5%) as the carbon source was performed. Fungal transfers and the addition of cellobiose and vitamins were made by using plastic syringes with hypodermic needles to pierce the butyl rubber septa. Fungi were stored at −70°C in 10 ml of culture broth containing 5% (vol/vol) dimethyl sulfoxide as a cryoprotectant (33).

Stock solutions were maintained on cellobiose (0.5%) or milled wheat straw (0.1%) in M2 medium. Inocula (5%) for growth experiments were cultured for 3 days at 39°C in M2 medium with cellobiose as the substrate.

Cell wall preparation. Cell walls were prepared from freshly harvested ryegrass (*Lolium perenne* cv. Tresor). The grass was separated into stem and leaf fractions by hand and lyophilized. Cell walls were prepared, as described previously (27), by using the neutral detergent system (except the use of amylase) described by Goering and Van Soest (11). The grass was cultivated in a field near Achterberg, The Netherlands, by CPRO-DLO (Wageningen, The Netherlands) and harvested at the end of June 1991 at a relatively young stage of growth.

Analysis. Chemical analysis and cell wall degradation were determined in triplicate. Cultures were harvested after 0, 1, 2, 3, 4, and 8 days of incubation. Supernatants were separated from residual particulate substrate and adherent fungal biomass by centrifugation at 10,000 × *g* for 15 min. Supernatants from three replicate cultures were analyzed for formate by high-performance liquid chromatography (HPLC) with differ-

* Corresponding author. Mailing address: ATO-DLO, P.O. Box 17, 6700 AA Wageningen, The Netherlands. Phone: 31 317 475213. Fax: 31 317 412260. Electronic mail address: L.Sijtsma@ATO.DLO.NL.

TABLE 1. Neutral sugar composition of leaf and stem cell walls of perennial ryegrass from cultures harvested from 1 to 8 days after inoculation with *Neocallimastix* sp. strain CS3b[a]

Day(s) since inoculation	Amt of neutral sugar (% dry wt)									
	Arabinose		Galactose		Glucose		Xylose		Total	
	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem
0	3.6	3.7	2.2	2.7	37.2	45.7	10.1	11.1	53.1	63.3
1	3.7	3.9	2.3	2.8	39.4	45.1	10.9	12.0	56.5	64.0
2	1.8	3.1	2.1	2.5	31.6	42.2	7.5	10.6	43.1	58.5
3	0.7	1.6	2.3	2.1	9.7	19.4	3.2	6.6	16.2	29.8
4	0.4	0.9	2.2	4.0	3.6	10.5	1.2	3.2	7.5	18.9
8	0.3	0.6	2.2	1.4	4.8	5.4	1.0	2.1	8.5	9.9

[a] The standard error was below 10%. In both leaf and stem cell walls, trace amounts of rhamnose were detected, whereas only stems contained trace amounts of mannose.

ential refractometer detection (pump, Waters 510; column, PRP-X300 [Hamilton Co., Reno, Nev.]; temperature, 30°C; eluent, 0.4 mM H_2SO_4; flow rate, 0.6 ml · min^{-1}; detector, Waters 410 differential refractometer; UV detection at 210 nm). Hydrogen in 0.5- to 1.0-ml headspace gas samples was determined with a Hewlett-Packard 427 gas chromatograph fitted with a thermal conductivity detector and a column packed with Molsieve 5A (27). Nitrogen was used as carrier gas. Cell wall degradation was measured by weighing pelleted cell walls after treatment with acid pepsin (0.2% pepsin in 0.08 M HCl; room temperature, 48 to 60 h) for the removal of adherent fungi (1). Cell walls were washed twice with 0.01 M phosphate buffer (pH 7) and before being weighed were lyophilized to constant weight. For monosaccharide analysis, lyophilized pellets (10 to 100 mg) were hydrolyzed with 12 M H_2SO_4 for 1 h at 30°C and subsequently boiled in 1 M H_2SO_4 for 3 h. The mixture was neutralized with $Ba(OH)_2$, and the neutral sugars were quantified by HPLC (Dionex CarboPak PA1 column) using pulsed amperometric detection (9, 10). The column was equilibrated with 15 mM NaOH for 8 min at 30°C and directly after injection eluted with milliQ for 40 min at a flow rate of 0.8 ml · min^{-1}. A mixture of glucose, galactose, xylose, arabinose, mannose, and rhamnose was used as a standard. Mannitol and fucose, added after hydrolysis, were used as internal standards.

Composition of undigested and digested stem and leaf cell walls. The combined cellulose and hemicellulose fraction was quantified from the amounts of the major monosaccharides (glucose, xylose, arabinose, and galactose) in lyophilized cell wall hydrolysates. Since fungal biomass may represent up to 20% of the residual cell wall (2), pepsin treatment was included to remove fungi (1). In the undigested substrate, the combined cellulose and hemicellulose fraction represented 53.1 (wt/wt) and 63.3% of the dry weight of perennial ryegrass leaf and stem cell walls, respectively. In leaf cell walls, the monosaccharide composition of the combined cellulose and hemicellulose fraction was 70.0% glucose, 19.0% xylose, 6.8% arabinose, and 4.1% galactose. For stem cell walls, the percentages were 72.2% glucose, 17.5% xylose, 5.8% arabinose, and 4.3% galactose (Table 1). In both leaf and stem cell walls, trace amounts of rhamnose were detected, whereas only stems contained trace amounts of mannose.

After 1 day of incubation, no effect of *Neocallimastix* sp. strain CS3b on cell wall composition was determined. Cell walls harvested after this period of fermentation contained a considerable smaller amount of neutral sugars compared with those of undigested cell walls. These results were more evident for arabinose, glucose, and xylose than for galactose. Furthermore, the sugar content of leaf cell walls decreased before a decline in the sugar content of stem cell walls was observed.

Cell wall digestion and product formation. Previously, we demonstrated that the growth of *Neocallimastix* sp. strain CS3b on grass cell walls results in a loss of cell wall dry weight and the production of fermentation end products, such as formate, acetate, lactate, ethanol, and hydrogen (27). In this study, we present the results for formate and hydrogen, which may be used as indicators for fungal growth (17, 20). After an initial lag period of about 1 day, the growth of *Neocallimastix* sp. strain CS3b was accompanied by the accumulation of fermentation end products formate and hydrogen and the removal of dry weight and neutral sugars from the cell walls of perennial ryegrass (Fig. 1). Within the first 2 days of fermentation, the levels of product formation and cell wall degradation were higher for leaf cell walls than for stem cell walls. During growth, a linear correlation could be used to describe the relationship between cell wall degradation and product formation. Formate accumulation can be described as 0.4 ($\pm$ 1.06) + 0.350 ($\pm$ 0.015) $\times$ digested cell walls ($r^2 = 0.985$; $n = 9$), and hydrogen accumulation can be described as 0.28 ($\pm$ 0.30) + 0.147 ($\pm$ 0.004) $\times$ digested cell walls ($r^2 = 0.995$; $n = 9$).

To compare losses in dry weight and neutral sugars, the results are presented as percentages of the corresponding amounts in undigested cell walls. The rate and final extent of neutral sugar removal were high compared with the loss of cell wall dry weight for both stem and leaf cell walls (Fig. 1). These differences may be attributed partly to the presence of an unfermentable fraction, which formed an increasing proportion of the cell wall dry weight with increased fermentation time.

In Fig. 2 the losses of the four major sugars (glucose, xylose, arabinose, and galactose) are shown. These data are presented on a percentage basis, as described above. No significant differences were found in the removal of glucose, xylose, and arabinose from leaf and stem cell walls of perennial ryegrass by *Neocallimastix* sp. strain CS3b. Galactose, however, was degraded to a lesser extent.

Data from formate and hydrogen production indicate that the growth of *Neocallimastix* sp. strain CS3b was completed in 4 days in the culture system used. During this period, the fungus liberated about 95% of the fermentable sugars in untreated material.

Our results demonstrate that *Neocallimastix* sp. strain CS3b was able to remove nearly all glucose, xylose, and arabinose and a considerable proportion of galactose from leaf and stem cell walls of perennial ryegrass (Table 1; Fig. 2). With galactose as an exception, these results are consistent with reports in which the anaerobic fungus *Neocallimastix* sp. strain R1 (30) and mixed populations of ruminal bacteria (21) have been shown to participate in the simultaneous removal of neutral sugars of cell wall constituents. It is not clear yet whether these constituents are removed as monosaccharide units or as polymers (or both). The high level of xylose removal from grass cell walls and the low level of galactose removal are in contrast with data of Theodorou et al. (30), who found similar removal rates for the four major sugars in Italian ryegrass hay but a significantly reduced extent of digestion for xylose when compared with that for arabinose, galactose, or glucose. Temperate forages consist of a heterogeneous population of cell types. Upon treatment with microorganisms, a trend toward increased xylose and decreased glucose contents has been observed (12). This trend, however, usually reflects the preferential degradation of primary cell walls, which have a lower xylose content, relative to that of secondary cell walls (13). The cell walls used

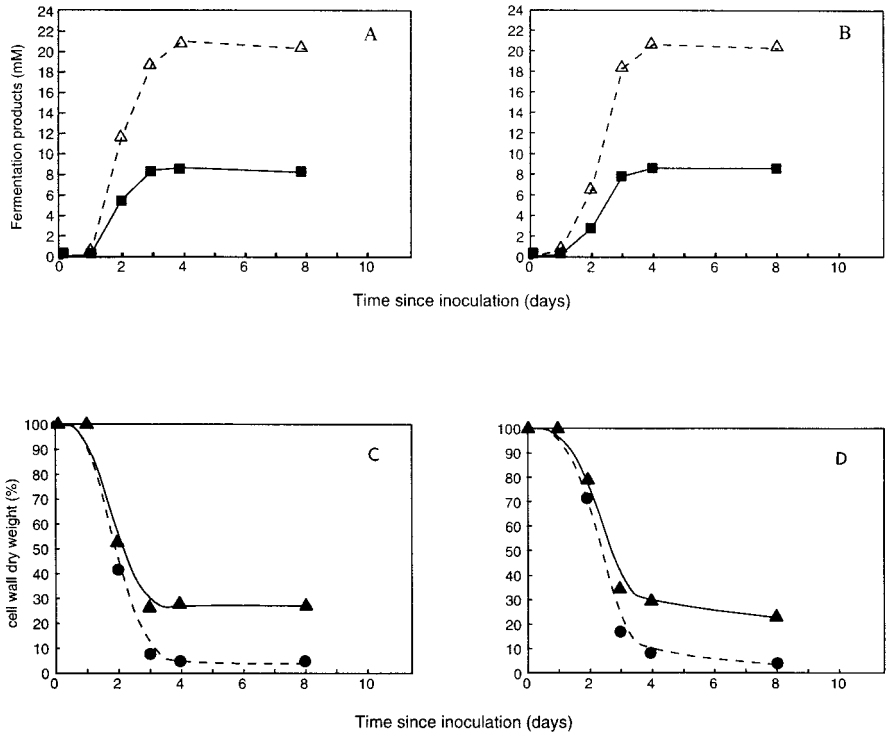

FIG. 1. Product accumulation (A and B) and cell wall dry weight and cell wall sugar (arabinose, galactose, glucose, and xylose) losses (C and D) during fermentation of leaf (A and C) and stem (B and D) cell walls of perennial ryegrass by *Neocallimastix* sp. strain CS3b. Symbols: △, formate; ■, hydrogen; ▲, residual cell walls; ●, residual sugars.

in this study were prepared from grass harvested at a relatively early growth stage. Therefore, the amount of thickened and lignified secondary cell walls may be limited in both leaf and stem cell walls, which may explain the great extent of degradation for whole cell walls and cell wall sugars.

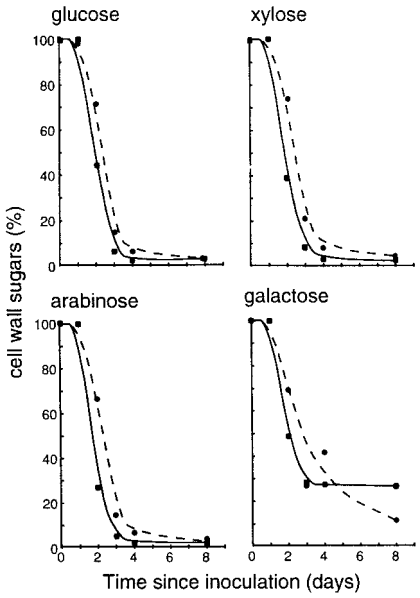

FIG. 2. Removal of individual sugars from leaf (■) and stem (●) cell walls isolated from perennial ryegrass during fermentation by *Neocallimastix* sp. strain CS3b.

Anaerobic fungi produce a complex of enzymes necessary for the degradation of plant cell walls (19, 25, 27, 29). However, reports suggest that anaerobic fungi are unable to utilize all of the reducing sugars which they liberate from plant cell walls. Soluble sugars have been shown to accumulate during wheat straw and xylan fermentation (19), and although glucose and xylose are utilized as energy-yielding substrates by *Neocallimastix* sp. strain R1, arabinose and galactose are not. Similar results have been found for *Neocallimastix* sp. strain CS3b (27). However, since the rumen is a complex ecosystem, the liberation of sugars from cell walls may benefit the growth of other rumen microorganisms.

Because of the enzymes rumen fungi produce and their physical properties, colonization of lignin-containing tissues by them would appear to result in a weakened plant residue with altered physical characteristics. Such alterations are likely to be important characteristics that influence animal performance.

The results presented in this study show that upon incubation with *Neocallimastix* sp. strain CS3b, grass cell walls and cell wall sugars are degraded to a great extent and used as carbon sources. Furthermore, during the first 3 days of fermentation, fungal degradation of leaf cell walls is slightly higher than degradation of stem cell walls.

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