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THE EFFECT OF ACETYL GROUPS ON THE HYDROLYSIS OF RYEGRASS CELL WALLS BY XYLANASE AND CELLULASE FROM *TRICHODERMA KONINGII*

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Key Word Index—*Lolium perenne*; Gramineae; ryegrass; cell walls; cellulase; cellobiohydrolase; endo-1,4-β-glucanase; xylanase; acetyl.

Abstract—Delignified ryegrass cell walls were effectively hydrolysed by a mixture of endo-1,4-β-glucanase and xylanase, but the rate and extent of hydrolysis was greater when the cellobiohydrolase part of the cellulase system was also present. Deacetylation of the xylan in the cell walls had a significant effect on the rate but not on the extent of hydrolysis of delignified cell walls. Deacetylation followed by endoglucanase–xylanase action resulted in a significant decrease in the proportion of xylose present in the residual cell walls. However, when cellobiohydrolase was acting in admixture with the endoglucanase–xylanase, it was the cellulose component of deacetylated cell walls that was preferentially hydrolysed. The proportion of galactose in the unhydrolysed fraction of the cell walls increased significantly after enzyme action by the cellobiohydrolase–endoglucanase–xylanase system.

INTRODUCTION

Acetyl groups are known to be present as substituents on various plant polysaccharides, notably, hemicelluloses and pectins. In a study on a hard-wood hemicellulose preparation [1] it has been concluded that the acetyl substituents are located on C-2 and C-3 of the xylose residues of the 1,4-β-linked linear xylan chain [2], and it is assumed by analogy that the acetyl groups found in other hemicelluloses can be assigned to similar positions [3].

The degrees of substitution reported are high. In hard-wood xylan [1] and in xylans in mature grasses [2] every second xylose residue may be acetylated, but in beech leaves during expansion and maturation every xylose residue may carry an acetyl substituent [3].

Acetylation could greatly impede enzymic hydrolysis by microorganisms, and it has been suggested that it could be an important factor influencing digestibility of the plant cell wall in the ruminant [4]. These claims were based on the results of digestibility tests carried out on dried grass inserted into the rumen in nylon bags, when it was found that xylose was the least digestible sugar, and that the proportion of acetyl increased in tandem with the xylose as digestion proceeded. In this paper cell-free cellulase and xylanase components from the fungus *Trichonderma koningii* have been used in an attempt to examine the effect of acetyl substituents on the degradation of grass cell walls by microorganisms, and to identify factors that might affect digestibility in the rumen.

RESULTS AND DISCUSSION

Xylanase (EC 3.2.1.32) is a constitutive enzyme in *T. koningii*. When cultured using virtually pure cellulose (cotton fibre) as the sole carbon source, it is synthesized, in good yield, along with the cellulase (EC 3.2.1.4) (cellobiohydrolase, endo-1,4-β-glucanase) β-glucosidase enzymes. For the purposes of the present investigation the enzyme system was fractionated into, (a) a highly purified cellobiohydrolase (EC 3.2.1.91) component [5], (b) a fraction containing high M_r endo-1,4-β-glucanase (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21), and (c) another fraction containing low M_r endo-1,4-β-glucanase [6]. Neither of these enzyme fractions acting alone could solubilize, to a significant extent, native highly ordered crystalline cellulose (e.g. cotton fibre): reconstituted mixtures of the enzymes, however, solubilized all forms of crystalline cellulose with ease.

Xylanase activity was found in the fraction containing high M_r endo-1,4-β-glucanase activity, but not in the fraction containing cellobiohydrolase. Only the cellobiohydrolase and the high M_r endo-1,4-β-glucanase–xylanase fractions were used in the present study.

Effect of acetyl groups on the independent action of cellobiohydrolase and endoglucanase-xylanase on delignified cell walls

Delignified ryegrass cell walls were not hydrolysed by purified cellobiohydrolase to any significant extent (Fig. 1), but hydrolysis of the neutral carbohydrate was extensive with the endoglucanase–xylanase portion of the enzyme system. The relative proportions of the neutral sugars present in the residual cell wall material left after enzymatic hydrolysis for 14 days by endoglucanase–xylanase was similar to that in the untreated cell wall (Table 1), and this demonstrates that neither the cellulose nor the xylan were limiting in this circumstance.

Delignified cell walls contained 1.7% acetyl. Removal of these acetyl substituents on the xylan chain with sodium methoxide resulted in a marked increase in rate but not in the extent of degradation of the cell wall polysaccharides to action of the endoglucanase–xylanase fraction when measured over 14 days (Fig. 1). Deacetylation resulted in a marked decrease (22% to

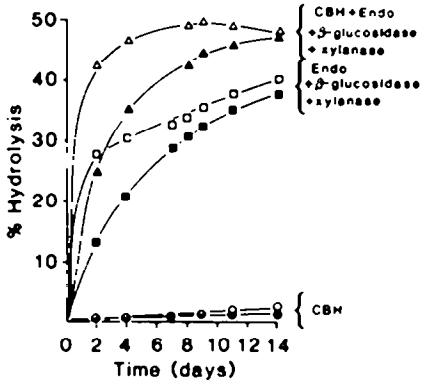

Fig. 1. Hydrolysis of ryegrass cell walls by xylanase and cellulase components of *T. koningii*. Details of the incubation and assay of soluble sugars released are given in the Experimental section. △, □ and ○, Deacetylated cell walls; ▲, ■ and ●, cell walls not deacetylated. CBH, Cellobiohydrolase; endo, endo-1,4-β-glucanase.

10%) in the proportion of xylose present in the residual cell walls after 14 days incubation (Table 2). In contrast, endoglucanase–xylanase action on cell walls that had not been deacetylated (Table 1) did not result in any change in the proportion of sugars in the residual cell walls, despite the fact that the degree of hydrolysis was similar to that effected on the deacetylated material (Fig. 1).

On the basis of these results it would appear that xylan became more susceptible to enzyme action after deacetylation with sodium methoxide.

The ratio of arabinose to xylose in the enzyme-treated deacetylated cell walls was higher (0.43) than in the control (0.32), indicating that arabinose residues were more resistant than xylose residues to enzyme action [7].

Effect of acetyl substituents on the combined action of cellobiohydrolase, endoglucanase and xylanase on delignified cell walls

Although cellobiohydrolase is an essential component of the cellulase system that can solubilize 'crystalline' highly ordered cellulose in cotton fibre [5, 8], its presence

Table 1. Sugar composition of delignified ryegrass cell walls before and after treatment with *T. koningii* cellulase and xylanase components

Sugar	No enzyme treatment	Enzyme treatment: Cellobiohydrolase	Endoglucanase + xylanase*	Cellobiohydrolase + endoglucanase + xylanase*
Rhamnose	0.99	0.59	0.67	1.40
Fucose	2.22	—	0.54	—
Arabinose	7.83	8.79	6.84	12.58
Xylose	22.97	24.27	22.34	31.48
Mannose	—	—	—	0.75
Galactose	2.85	2.91	4.16	8.69
Glucose	63.15	63.74	66.06	45.49

*These fractions also contained β-glucosidase activity.

Table 2. Sugar composition of delignified-deacetylated ryegrass cell walls before and after treatment with *T. koningii* cellulase and xylanase components

Sugar	No enzyme treatment	Enzyme treatment: Cellobiohydrolase	Endoglucanase + xylanase*	Cellobiohydrolase + endoglucanase + xylanase*
Rhamnose	0.41	—	0.78	4.15
Fucose	—	—	0.72	
Arabinose	7.18	8.02	4.52	15.33
Xylose	22.4	23.2	10.50	26.17
Mannose	—	—	1.03	—
Galactose	2.41	2.42	2.71	11.10
Glucose	67.77	65.84	81.01	43.26

*These fractions also contained β-glucosidase activity.

did not appear to be necessary for hydrolysis of cellulose in the cell walls of delignified perennial ryegrass. Indeed, as already indicated, extensive hydrolysis could be effected by the action of the endoglucanase–xylanase fraction acting independently (Fig. 1). However, when cellobiohydrolase was mixed with the endoglucanase–xylanase fraction to give a complete enzyme system, there was a significant increase in the rate and extent of hydrolysis, and this, presumably, was because the crystalline cellulose component was now also being hydrolysed. Deacetylation caused a marked increase in the rate of hydrolysis by the complete enzyme system (Fig. 1), but after 14 days incubation the degree of hydrolysis was approximately the same as cell walls that had not been deacetylated.

The ratio of glucose to xylose in the enzyme-treated cell walls (deacetylated, 1.65; non-deacetylated, 1.44) was considerably less than in the control (2.75) (Tables 1 and 2), indicating that a larger proportion of the cellulose was being hydrolysed; and this was to be expected. What was not expected was the 3- and 4-fold increases in the proportion of the galactose present, without and with deacetylation, respectively.

The disaccharide *O*-β-D-galactopyranosyl-1,4-D-xylose has been noted in acid hydrolysates of some xylans and it has been suggested that these may derive from *O*-β-D-galactopyranosyl-1,4-*O*-β-D-xylopyranosyl-1,2-L-arabinofuranose side chains [9]. If side chains similar to those occur in the xylans of perennial ryegrass, albeit in small amounts, the present results would suggest that they are relatively resistant to enzymatic hydrolysis by *T. koningii* xylanase and perhaps to other xylanase systems. It is possible that such side chains may be important in controlling digestibility of the plant cell wall in the ruminant.

EXPERIMENTAL

Plant material. Perennial ryegrass was cut *ca* 5 cm from the ground from established pastures at the East of Scotland Agricultural College, Craibstone, Aberdeen. It was freeze dried immediately, hammermilled (2 mm), extracted with methylated spirits in a Soxhlet extractor and dried in air. Delignification with HOAc–$NaClO_2$ was as described in ref. [10].

Enzyme preparation and fractionation. Cultures of *Trichoderma koningii* IMI 73022 were prepared using cotton fibres as the sole carbon source, harvested and concd as previously described [11]. The cellulase and xylanase enzymes were fractionated by gel filtration on Ultrogel AcA44 [11] and on DEAE-Sepharose [11] into a low M_r endo-1,4-β-glucanase, a cellobiohydrolase and another endo-1,4-β-glucanase component associated with xylanase and β-glucosidase activity. Purified cellobiohydrolase was obtained by isoelectric focusing in an LKB isoelectric focusing column using ampholyte covering the pH range 3–6 [5].

Incubation of cell walls with enzymes. Delignified cell walls (20 mg) were incubated at 37° for 14 days with enzyme [cellobiohydrolase, 200 μg; endo-1,4-β-glucanase (1000 units CM-cellulase) [12]], in a reaction mixture consisting of 5 ml of 0.2 M NaOAc buffer, pH 4.8, 0.2 ml of 0.05 M NaN_3, and H_2O and enzyme to give a total vol. of 10 ml. At intervals several tubes were removed and the residues isolated by centrifugation. The unhydrolysed cell walls were washed with H_2O and freeze dried. The supernatant were analysed for total sugar using the phenol-H_2SO_4 method [13].

Analysis of neutral carbohydrate of cell walls. Acid hydrolysis was performed as described in ref. [14]. After addition of inositol as internal standard, the neutral sugars in hydrolysates were determined by GC on a Pye Model 104 gas chromatograph, using a glass column (1.5 m × 6.0 mm) of 5% SR 2340 coated on Gas-Chrom Q.

Determination of acetyl. Acetyl groups were determined by GC as the benzyl ester after extraction with NaOMe [15].

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