Phytochemistry, 1973, Vol. 12, pp. 763 to 766. Pergamon Press. Printed in England.

COMPARISON OF CELL-WALLS OF *LOLIUM MULTIFLORUM* WITH COTTON CELLULOSE IN RELATION TO THEIR DIGESTION WITH ENZYMES ASSOCIATED WITH CELLULOLYSIS*

R. D. HARTLEY and E. C. JONES
Grassland Research Institute, Hurley, Berks.

and

T. M. WOOD
Rowett Research Institute, Bucksburn, Aberdeen

(*Revised received* 26 *October* 1972. *Accepted* 13 *November* 1972)

Key Word Index—*Lolium multiflorum*; Gramineae; cell-walls; cellulolysis; lignin-carbohydrate linkages; ferulic acid.

Abstract—Activities of several enzymes associated with cellulolysis were compared using as substrates cell-walls of *Lolium multiflorum* and cotton cellulose. Purified enzymes C_1 (see Ref. 1 for definition), C_x (CM-cellulase) and β-glucosidase were employed as well as culture filtrates containing C_x. Activities were determined by ability to digest the substrates and to release H_2O-soluble phenolic compounds from the grass cell-walls. The culture filtrates most active on cotton cellulose were obtained using the fungi *Trichoderma viride* and *Fusarium solani*; with grass cell-walls the most active were from *T. viride*, *Gliocladium roseum*, a species of Basidiomycetes, and one strain of *Myrothecium verrucaria* (IMI Strain 25 291). For the crude enzyme preparations tested, there were highly significant correlations between the digestibility of grass cell-walls and the UV-absorption of the filtrate at λ_{max} 290 nm and at λ_{max} 324 nm but there was no significant correlation between the digestibility of grass cell-walls and that of cotton cellulose. Partially purified C_1 and C_x from two different fungal sources showed activity on both substrates. Differences in MW of the H_2O-soluble phenolic compounds obtained by treatment of grass cell-walls with C_1 and C_x components suggest that these enzymes could have different modes of action. Synergism between C_1 and C_x from *T. koningii* occurred with both substrates but with C_1 and C_x from *F. solani* synergism only occurred with cotton cellulose.

INTRODUCTION

IN THE previous paper,[2] it was shown that treatment of ryegrass cell-walls with a commercial crude cellulase preparation caused the release of H_2O-soluble carbohydrate esters of ferulic acid. Since such esters might arise from the covalent association of lignin and carbohydrate in cell-walls, it is apparent that this mechanism of degradation could be of considerable importance in the breakdown of organic matter in the alimentary tract of the ruminant animal or in soil. This disruption of the structural integrity of the cell-wall is likely to cause an increase in the availability of the structural carbohydrates to micro-organisms.

Whereas culture filtrates from all cellulolytic microorganisms contain C_x-enzymes (CM-cellulases), relatively few contain the C_1 component. This C_1 component has recently been shown to be a cellobiohydrolase which acts synergistically with the random-acting

* Part II in the series "Lignin-carbohydrate linkages in plant cell-walls". For Part I see Ref. 2.

[1] REESE, E. T., SIU, R. G. H. and LIEVINSON, H. S. (1950) *J. Bact.* **59**, 485.
[2] HARTLEY, R. D. (1973) *Phytochem.* **12**, 661.

C_x components in accomplishing the digestion of native cellulose.[3] In the present paper, the activities of the enzymes C_1, C_x and β-glucosidase from various fungal sources have been compared using both grass cell-walls and cotton cellulose as substrates. The MWs of the phenolic products resulting from the treatment of grass cell-walls with the C_1 and the C_x components have also been determined and the results compared.

RESULTS AND DISCUSSION

The activities of culture filtrates from various fungal sources using cotton and grass cell-walls as substrates, are shown in Table 1. All the filtrates had C_x activity but only those from *Trichoderma viride*, *Sporotrichum pruinosum* and *Fusarium solani* also had C_1 present in sufficient quantity to cause extensive digestion of cotton. In general, the grass cell-walls were more susceptible to attack by the culture filtrates than the cotton and therefore digestions of cotton were carried out with higher enzyme concentrations and longer incubation times. There was no significant correlation between the digestibilities of the cotton and grass cell-walls which are listed in Table 1, and in some cases there were considerable variations. For example, *F. solani* culture filtrate was highly active on cotton but one of the least active on grass cell-walls. With the grass cell-walls as substrate, the filtrate from *Myrothecium verrucaria* strain 25 291 was considerably more active than filtrates from other strains of this fungus that were tested.

TABLE 1. RELATIONSHIPS OF COTTON CELLULOSE AND GRASS CELL-WALL DIGESTIBILITIES WITH UV-ABSORBANCE OF WATER-SOLUBLE PRODUCTS FROM TREATMENT OF CELL-WALLS WITH CRUDE ENZYMES

Fungus	Strain IMI	Cotton cellulose Digested/ 500 units C_x (%)	Grass cell-walls Digested/ 25 units C_x (%)	Grass cell-walls A of filtrate*/ 25 units C_x at λ_{max} 324 nm
Trichoderma viride†		71	19·7	0·508
Memnionella echinata	24 287	0	6·7	0·111
Myrothecium verrucaria	25 291	5	15·0	0·247
Sporotrichum pruinosum	74 692	35	11·3	0·319
Fusarium solani	95 994	61	6·7	0·122
Gliocladium roseum	102 020h	0	19·3	0·606
Basidiomycete‡		17	18·0§	0·450§

* Filtrate diluted to 20 ml for measurement of absorbance (A) using 1 cm cell.
† Culture collection U.S. Army, Natick Labs., Natick, Mass., U.S.A. (Strain QM 9123).
‡ Crude cellulase preparation supplied by E. Merck and contained 370 units C_x/mg.
§ Maximum values of A were obtained using crude cellulase (*ex* Basidiomycete, *ca.* 2000 units C_x) and was 1·5 at λ_{max} 324 nm. At this stage the % digested was 71.

The treatment of similar grass cell-walls with a crude commercial cellulase preparation (*ex* Basidiomycete) was examined in detail earlier.[2] Under the conditions used in the present work (see Table 1), the commercial cellulase was, by comparison with the culture filtrates, highly active on grass cell-walls but only moderately active on cotton. It might be expected that the commercial cellulase would be more effective than the culture filtrates for the digestion of grass cell-walls as the Basidiomycetes contain many wood rotting fungi which can produce extracellular enzymes with hemicellulase as well as cellulase activity: grass cell-walls, like wood cell-walls, are rich in hemicelluloses.

[3] WOOD, T. M. (1972) *Biochem. J.* **128,** 1183.

The UV spectra of the filtrates from treatment of grass cell-walls with the commercial cellulase or with culture filtrates, were very similar. Major peaks were at 290 and 324 nm and earlier work[2] using the commercial cellulase had shown that these peaks were due to carbohydrate esters of ferulic acid. There were correlations between the digestibility of the grass substrates and absorbance (A) at λ_{max} 290 nm ($r = 0{\cdot}93$, $p < 0{\cdot}001$) and at λ_{max} 324 nm ($r = 0{\cdot}93$, $p < 0{\cdot}001$) for the 7 samples listed in Table 1 plus a further 5 samples from different strains of *M. verrucaria*.

The action of partially purified enzymes on cotton cellulose and grass cell-walls was examined by measuring digestibilities and A values. C_x from *F. solani* was much less active on cotton compared with the original culture filtrate which contained both C_1 and C_x.[4] This C_x sample also showed low activity on grass cell-walls (25 units C_x employed, 2·7% digested). In contrast to this behaviour, purified low MW C_x and C_x (containing some β-glucosidase) enzymes from *Trichoderma koningii*[5] were not very active on cotton but highly active on grass cell-walls. The two purified samples of C_1 showed activity at a low level on both substrates.

Synergism between C_1, C_x and β-glucosidase was investigated using the partially purified samples from *F. solani* and *T. koningii*. As reported earlier,[4,5] there was considerable synergism between C_1 and C_x using cotton substrates. With the grass substrate, there was no synergism between any of the three enzymes from *F. solani* but there was synergism between either the low MW C_x or C_x + β-glucosidase from *T. koningii* and the corresponding C_1 (Table 2). This synergistic effect was less marked than for the corresponding cotton substrate.

TABLE 2. SYNERGISM BETWEEN *Trichoderma koningii* CELLULASE COMPONENTS USING COTTON CELLULOSE AND GRASS CELL-WALLS AS SUBSTRATES

	Cotton cellulose				
	Amount of enzyme				
Enzymes present	C_1 (mg protein)	C_x (units)	β-Glucosidase (units)	Low MW C_x (units)	% digested
C_1	0·18				6
C_x + β-glucosidase		500	51		5
Low MW C_x				500	5
C_1 + C_x + β-glucosidase	0·18	500	51		74
C_1 + low MW C_x	0·18			500	24
C_x + β-glucosidase + low MW C_x		500	51	500	5
C_1 + C_x + β-glucosidase + low MW C_x	0·18	500	51	500	4

	Grass cell-walls					
	Amount of enzyme					
Enzymes present	C_1 (mg protein)	C_x (units)	β-Glucosidase (units)	Low MW C_x (units)	% digested	A of filtrate at λ_{max} 324 nm*
C_1	0·18				5·7	0·055
C_x + β-glucosidase		5·0	0·51		9·0	0·321
Low MW C_x				5·0	11·3	0·371
C_1 + C_x + β-glucosidase	0·18	5·0	0·51		23·4	0·438
C_1 + low MW C_x	0·18			5·0	24·5	0·469
C_x + β-glucosidase + low MW C_x		5·0	0·51	5·0	13·2	0·509
C_1 + C_x + β-glucosidase + low MW C_x	0·18	5·0	0·51	5·0	32·8	0·635

* Filtrate diluted to 20 ml (1 cm cell). For maximum values of A obtained with crude cellulase (*ex* Basidiomycete), see footnote of Table 1.

A comparison was made of the MWs and TLC of the H_2O-soluble phenolic compounds obtained by treatment of grass cell-walls with C_1 and with C_x + β-glucosidase enzymes (*ex T.*

[4] WOOD, T. M. (1969) *Biochem. J.* **115**, 457.
[5] WOOD, T. M. (1968) *Biochem. J.* **109**, 217.

koningii). The results showed that when conditions were adjusted to give filtrates having approximately 25% of the maximum A at λ_{max} 324 nm, the C_1 filtrate had 24·7% of the phenolic compounds with MW > 50 000 and 35·2% with MW < 700 compared with < 8% with MW > 50 000 and 27·1% with MW < 700 for the corresponding C_x + β-glucosidase filtrate. The MWs which were determined by gel chromatography may be low due to phenolic absorption causing high K_{av} values. When enzymes containing C_x were used, the filtrates contained 4 phenolic compounds of R_f 0, 0·14, 0·30 and 0·43, whereas the C_1 filtrate gave R_f 0, 0·13, 0·18 and 0·30. These results could be indicative of a difference in the mode of action of C_x and C_1. Sephadex gel chromatography of the filtrate from crude cellulase *ex* Basidiomycete (1850 units C_x) indicated that the % of phenolic compounds having MW < 700 was about twice that obtained with 5 units of C_x. This behaviour is consistent with higher concentrations of C_x causing greater degradation leading to the production of lower MW phenolic compounds.

Earlier work[2] with crude cellulase had not ruled out the possibility that the esters of ferulic acid are attached to protein in grass cell-walls although none of the esters was released from walls by treatment with pronase or Cleland's reagent. Therefore the possibility was investigated of either purified C_1 or C_x having proteolytic activity which would lead to the release of esters of ferulic acid. Filtrates from treatments of the cell-walls with either enzyme were examined by TLC and developed with ninhydrin but no release of proteinaceous material could be detected.

Of the four main phenolic compounds obtained by treatment of grass cell-walls with crude cellulase and separated by TLC, the phenol of R_f 0·43 had been shown previously to be a carbohydrate ester of ferulic acid in which the carbohydrate moiety was composed of xylose, arabinose and glucose units.[2] It is possible that the ester is attached to the cellulose forming part of the cell-wall and that it is liberated by degradation of the cellulose. Another possibility is that the ester is liberated from the hemicellulose fraction by the hydrolysis of β (1 → 4) glycosidic linkages in xylans by C_x itself or by hemicellulase contaminants. These possibilities are at present under investigation.

EXPERIMENTAL

Sources of enzymes. The method of Wood[5] was employed for the preparation of culture filtrates. The source of crude cellulase (*ex* Basidiomycete) has been given earlier.[2] Purified C_1, C_x and β-glucosidase were obtained as described previously.[4,5]

Methods. Measurement of C_1, C_x and β-glucosidase concentrations, sources of substrates, incubation procedures, and estimation of substrate digested have been described previously.[2,4,5] Sample 3 (*M. verrucaria*, Table 1) was concentrated 4-fold by evaporation at 30° before digestion assay. All UV-absorption measurements were corrected for A due to the enzymes used.

MW determination and TLC examination of H_2O*-soluble phenolic compounds obtained by treatment of grass cell-walls with cellulolytic enzymes* (ex T. koningii). Incubation of cell-walls was carried out in 30 mg batches using the above method with C_1 (0·18 mg protein) and with C_x (5 units) + β-glucosidase (0·51 units) to obtain filtrates of *ca.* 25% of maximum A at λ_{max} 324 nm (see footnote of Table 1). For C_1 the time of incubation was 2 weeks instead of the normal 16 hr. C_x (1000 units) + β-glucosidase (102 units)/30 mg cell walls were employed to obtain filtrates having maximum A at λ_{max} 324 nm. The percentages of phenolic compounds in the filtrates having MW < 700 were calculated from their A at λ_{max} 324 nm and from A of their eluates of $K_{av} = 0$ on Sephadex G10. Similarly phenolic compounds having MW > 50 000 were calculated from A of similar eluates from G75. Filtrates were examined by TLC on cellulose using *n*-BuOH–HOAc–H_2O (62:15:23) with *p*-anisidine phthalate and Fast Blue B spray reagents, as previously described.[2] Ninhydrin reagent for the development of TLC plates was prepared by the method of Brenner *et al.*[6]

Acknowledgements—The authors wish to thank Messrs. M. A. Chaudhry and A. T. Masters for technical assistance, and Dr. L. H. P. Jones for his interest in the course of this work.

[6] BRENNER, M., NIEDERWIESER, A. and PATAKI, G. (1969) in *Thin-layer Chromatography* (edited by STAHL, E.), 2nd Edn, p. 747, Springer, Berlin.