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# Purification and Partial Characterization of Two Feruloyl Esterases from the Anaerobic Fungus *Neocallimastix* Strain MC-2

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**Two extracellular feruloyl esterases (FAE-I and FAE-II) produced by the anaerobic fungus *Neocallimastix* strain MC-2 which cleave ferulic acid from *O*-{5-*O*-[(*E*)-feruloyl]-α-L-arabinofuranosyl}-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX) were purified. The molecular masses of FAE-I and FAE-II were 69 and 24 kDa, respectively, under both denaturing and nondenaturing conditions. Apparent $K_m$ and maximum rate of hydrolysis with FAXX were 31.9 μM and 2.9 μmol $min^{-1}$ $mg^{-1}$ for FAE-I and 9.6 μM and 11.4 μmol $min^{-1}$ $mg^{-1}$ for FAE-II. FAE-II was specific for FAXX, but FAE-I hydrolyzed FAXX and PAXX, the equivalent *p*-coumaroyl ester, at a maximum rate of metabolism ratio of 3:1.**

The hemicellulosic fraction of forages and cereals is composed mainly of xylan formed from β-1,4-linked xylopyranosyl residues. Xylans found in forages are extensively substituted with acetyl, uronyl, and arabinosyl residues. In grasses, 40 to 50% of the arabinosyl residues are substituted at the *O*-5 position (9), and a portion of these arabinosyl groups are ester linked to *p*-coumaroyl and feruloyl moieties (14). There is evidence that these esterified phenolic groups dimerize in vivo either by oxidative coupling, forming dehydrodiferulic acid (15, 18), or by photodimerization in sunlight, forming a series of substituted truxillic and truxinic acid cross-links (16). Evidence also suggests that feruloyl and *p*-coumaroyl groups form bonds between the xylan heteropolymer and lignin (9, 21, 23).

Hindrance of plant cell wall biodegradation appears to be associated with in situ structural relationships between different types and quantities of cross-linking bonds between cell wall polymers rather than to any one specific cell wall component (11, 12). The extent of cross-linking by ferulic and *p*-coumaric acid dimers appears to correlate best with low biodegradability (12, 14). Furthermore, the esterification of feruloyl and *p*-coumaroyl groups to arabinoxylans has been shown to limit the release of cell wall pentoses (1, 17).

Anaerobic rumen fungi preferentially colonize and degrade lignified cell walls, which are the least biodegradable plant components (2). These fungi produce high levels of both *p*-coumaroyl and feruloyl esterases (3, 6), and a *p*-coumaroyl esterase has been purified to homogeneity from the anaerobic fungus *Neocallimastix* strain MC-2 (7). Feruloyl, but not *p*-coumaroyl, esterase has been reported in culture supernatants of *Schizophyllum commune* (22), *Aspergillus niger* (19), and *Streptomyces olivochromogenes* (20), from which it has been recently purified (10). The present work reports the purification and partial characterization of two extracellular feruloyl esterases, FAE-I and FAE-II, from the anaerobic fungus *Neocallimastix* strain MC-2.

*Neocallimastix* strain MC-2 was isolated and maintained as previously described (4). For enzyme production the fungus was grown in four separate 2-liter autoclave bottles (AMSCO) containing 1.75 liters of basal media and cellulose (MN-300, 2% [wt/vol]) as substrates for 5 days at 39°C (7). Although *Neocallimastix* strain MC-2 produces more feruloyl esterase from growth on plant cell walls (6), cellulose was used as a growth substrate in the present investigation to avoid protein aggregation that occurs with plant cell wall compounds (7) during purification of the esterases.

Assays for feruloyl and *p*-coumaroyl esterases were performed at 40°C and at pH 6.8, as previously described, by using as substrates *O*-{5-*O*-[(*E*)-feruloyl]-α-L-arabinofuranosyl}-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX) and *O*-{5-*O*-[(*E*)-*p*-coumaroyl]-α-L-arabinofuranosyl}-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (PAXX) (150 μM) isolated from coastal Bermuda grass (*Cynodon dactylon*) (CBG) cell walls (Fig. 1) (5, 7). A similar assay was used for determining activity towards the feruloyl tetrasaccharide substrates {5-*O*-[(*E*)]-feruloyl]-[*O*-β-D-xylopyranosyl-(1→2)]-*O*-α-L-arabinofuranosyl-[1→3]}-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose ($FAX_3$-I; 150 μM) and *O*-β-D-xylopyranosyl-(1→4)-*O*-{5-*O*-[(*E*)-feruloyl]-α-L-arabinofuranosyl-[1→3]}-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose ($FAX_3$-II; 150 μM) isolated from CBG cell walls (13). The ability of purified feruloyl esterases FAE-I and FAE-II to release feruloyl moieties from ground CBG cell walls and assay methods for methyl ferulate esterase, xylosidase, arabinosidase, acetyl-esterase, and acetyl xylan esterase were previously reported (4, 6, 7). One unit of enzyme activity was defined as the amount which released 1 μmol of product $min^{-1}$, and specific activity is given in units per milligram of protein. Protein was determined by the method of Bradford (8). The pH optima and the effect of temperature on the purified feruloyl esterases were determined by using FAXX as the substrate. The effect of substrate concentration on feruloyl esterase activity was examined at 40°C in bis tris propane buffer {1,3-bis[tris(hydroxymethyl)-methylamino]propane} (BTP; at optimum pH for each esterase) with FAXX at concentrations that varied between 0.1 and 100 μg $ml^{-1}$. $K_m$ and maximum rate of

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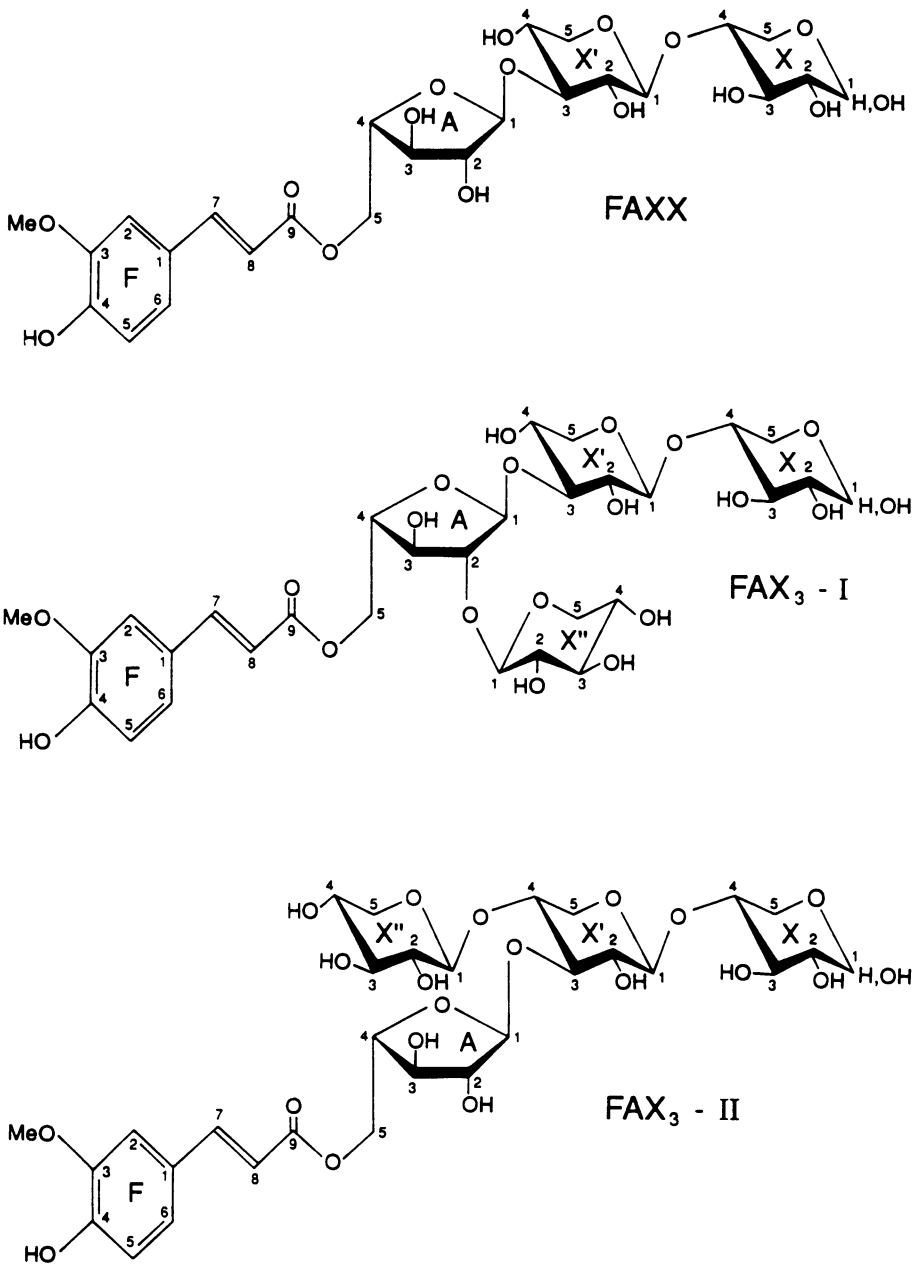


FIG. 1. Structure of feruloyl oligosaccharides purified from CBG (*Cynodon dactylon*) cell walls. PAXX is equivalent to FAXX containing a *p*-coumaroyl instead of feruloyl esters.

hydrolysis ($V_{max}$) values were calculated by the double-reciprocal method of Lineweaver and Burk.

Purification of FAE-I and FAE-II was performed at 4°C and is summarized in Table 1. After 5 days of growth, the culture supernatant (7 liters) was filtered (50-mesh nylon) to remove residual cellulose and fungal biomass. The retentate was washed with histidine buffer (1 liter, 50 mM, pH 6.0) and then with distilled water (1 liter). Culture supernatant and retentate washings were combined and concentrated by tangential flow ultrafiltration (Pellicon Cassette System with PTGC 10,000 NMWL cassette; Millipore, Bedford, Mass.), and the retentate was dialyzed against histidine buffer (5 mM, pH 6.0) containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.02% (wt/vol) sodium azide. The final concentrate was centrifuged (20,000 × *g*) for 20 min to sediment large particulate matter, and the supernatant was then ultracentrifuged (100,000 × *g*) for 1 h. The resultant supernatant from ultracentrifugation contained greater than 99% of feruloyl esterase activity.

The concentrated supernatant was subjected to isoelectric focusing in a Rotofor preparative isoelectric focusing cell (Bio-Rad, Richmond, Calif.). The sample was focused by using 2% (wt/vol) ampholytes (Biolyte pH 3-10; Bio-Rad), 3 M urea, and glycerol (10% [vol/vol]) in the Rotofor cell at 4°C for 5 h at 12-W constant power until the voltage and current remained constant (start conditions, 300 V and 19

TABLE 1. Purification of feruloyl esterases from *Neocallimastix* strain MC-2

| Purification step and feruloyl esterase | Total protein (mg) | Total activity (U)[a] | Sp act (U $mg^{-1}$) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Culture supernatant | 880 | 48.4 | 0.055 | 100 | 1.0 |
| Concentrate | 554.4 | 48.3 | 0.087 | 99 | 1.6 |
| Isoelectric focusing (Rotofor) | 34.8 | 68.1 | 0.511 | 72 | 9.3 |
| Superose 12 HR (prep grade) | | | | | |
| FAE-I | 16.6 | 11.1 | 0.67 | 58[b] | 34[b] |
| FAE-II | 14.3 | 17.0 | 1.19 | | |
| Mono Q | | | | | |
| FAE-I | 9.6 | 9.9 | 1.03 | 49[b] | 55[b] |
| FAE-II | 6.8 | 13.8 | 2.01 | | |
| Superose 12 HR + Superdex 75 HR | | | | | |
| FAE-I | 2.0 | 6.2 | 3.09 | 33[b] | 257[b] |
| FAE-II | 0.87 | 9.8 | 11.2 | | |

[a] Units are defined as micromoles of feruloyl groups released from FAXX per minute at 40°C and optimum pH.
[b] Value is derived from total of FAE-I and FAE-II protein and activity at each separation step.

mA; end conditions, 1,100 V and 8 mA). Fractions of 2.5 ml were collected. Fractions 4 to 8 (pH 4.23 to 5.78) containing feruloyl esterase activity were combined and concentrated to 1.5 ml by using a centrifugal ultrafiltration device (Centriprep, 10-kDa cutoff; Amicon, Beverly, Mass.). Sample aliquots (0.5 ml) were applied to a column of Superose 12 (prep grade) (HR 16/50, 1.6 by 50 cm; Pharmacia LKB Biotechnology, Piscataway, N.J.) connected to a fast protein liquid chromatography (Pharmacia) system. The column was equilibrated with BTP buffer (50 mM, pH 6.8) containing 5% (wt/vol) betaine to help minimize protein aggregation (7), 150 mM NaCl, 0.02% (wt/vol) sodium azide, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The enzymes were eluted at a flow rate of 0.5 ml $min^{-1}$, and fractions of 1 ml were collected. Two peaks of differing molecular weights containing feruloyl esterase activity were separated. High (FAE-I)- and low (FAE-II)-molecular-weight fractions containing feruloyl esterase activity were independently pooled and dialyzed against BTP buffer (20 mM, pH 6.8), 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. FAE-I and FAE-II fractions were individually loaded on a Mono Q (HR 10/10, 1 by 10 cm; Pharmacia) anion-exchange column and eluted with a linear gradient of NaCl (0 to 0.5 M) in 20 mM BTP buffer. A majority of the activity from the FAE-I fraction eluted at 0.34 M NaCl, and a majority of the FAE-II fraction eluted at 0.20 M NaCl. Attempts to separate the feruloyl esterases by anion-exchange chromatography prior to the Superose gel filtration step resulted in multiple active fractions. Fractions containing feruloyl esterase activity were concentrated to 1.5 ml as described above. Sample aliquots (0.5 ml) were applied to a Superose 12 (HR 10/30, 1.0 by 30 cm; Pharmacia) connected in series with a Superdex 75 (HR 10/30, 1.0 by 30 cm; Pharmacia) and eluted at 0.3 ml $min^{-1}$ as described above. High-performance gel filtration separated both FAE-I and FAE-II from trace protein contaminants which remained after chromatography on Mono Q. The ratio of FAE-I to FAE-II varied less than 3% for three separate purifications, suggesting that FAE-II is not a proteolytic product of FAE-I. Rumen microorganisms are known to secrete multiple forms of hydrolytic enzymes (9, 12). Subsequent to gel filtration, sodium dodecyl sulfate-(12.5%) polyacrylamide gel electrophoresis (SDS-PAGE) and staining with Coomassie R-250 showed homogenous protein bands corresponding to 69 and 24 kDa for FAE-I and FAE-II, respectively (Fig. 2). The molecular mass of FAE-II is similar to the 29 kDa found for a purified feruloyl esterase from *S. olivochromogenes* (10). High-performance gel filtration of the purified esterases (Fig. 3) revealed molecular masses of 68 and 24 kDa for FAE-I and FAE-II, respectively. These findings suggest that both feruloyl esterases are monomeric, whereas *p*-coumaroyl esterase isolated from the same fungal strain exists as a dimer of two polypeptides, each with a molecular mass of 5.8 kDa (7). The isoelectric points were determined to be 4.2 for FAE-I and 5.7 for FAE-II, both of which are considerably lower than the pIs of 7.9 and 8.5 observed for the feruloyl esterase from *S. olivochromogenes* (10).

FAE-I was most active in the pH range between 5.5 and 6.8, with the optimum at pH 6.2. FAE-II was most active in the pH range between 6.4 and 7.6, with the optimum at 7.0. Both esterases were relatively stable at 40°C under assay conditions. When incubated at 40°C for 8 h in the absence and presence of FAXX, FAE-I retained 37 and 90% activity

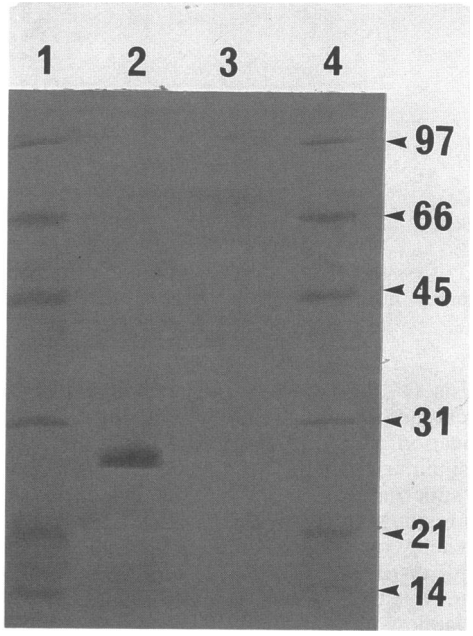


FIG. 2. SDS-PAGE of purified FAE-I (lane 3) and FAE-II (lane 2) and molecular mass protein standards in kilodaltons (lanes 1 and 4).

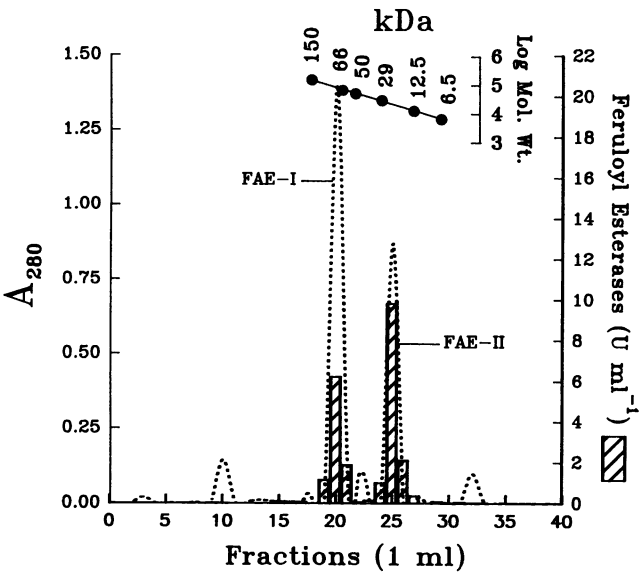


FIG. 3. Superose 12 plus Superdex 75 gel filtration chromatography of purified FAE-I and FAE-II. The column was equilibrated with 50 mM BTP buffer (pH 6.8) containing 5% (wt/vol) betaine, 150 mM NaCl, and 0.02% (wt/vol) sodium azide at a flow rate of 0.3 ml min$^{-1}$. The column was calibrated with the following, indicated by solid circles: sheep immunoglobulin G, 150 kDa; bovine serum albumin, 66 kDa; Fab fragment from immunoglobulin G, 50 kDa; carbonic anhydrase, 29 kDa; horse heart cytochrome *c*, 12.5 kDa; and bovine lung aprotinin, 6.5 kDa.

and FAE-II retained 28 and 71% feruloyl esterase activity, respectively. Glycerol (15% [vol/vol]) was added to the Superdex column eluant buffer containing the purified enzymes, and they were stored at −80°C. More than 80% of original activity was retained over a period of 2 months.

The purified FAE-I and FAE-II exhibited Michaelis-Menten kinetics. The $K_m$ and $V_{max}$ values for FAXX at optimum pH and 40°C were 31.9 μM and 2.9 μmol min$^{-1}$ mg$^{-1}$ and 9.6 μM and 11.4 μmol min$^{-1}$ mg$^{-1}$ for FAE-I and FAE-II, respectively. The substrate specificity of FAE-I and FAE-II towards various plant cell wall phenolic-carbohydrate esters and other carbohydrates was examined (Table 2). FAE-I and FAE-II released feruloyl groups from feruloyl tri- and tetrasaccharides but at different rates. The addition of a 1→4-linked xylose residue to the X′ of FAXX (i.e., $FAX_3$-II [Fig. 1]) reduced the specific activity by 18 and 16% for FAE-I and FAE-II, respectively, compared with activity toward FAXX. In contrast, a xylose residue 1→2 linked to the arabinose of FAXX (i.e., $FAX_3$-I [Fig. 1]) increased specific activity by 21 and 23% for FAE-I and FAE-II, respectively. Analogous results had been observed with *p*-coumaroyl esterase for the hydrolysis rates of $PAX_3$-I and $PAX_3$-II compared with that of PAXX hydrolysis (7). FAE-I exhibited substantial activity toward PAXX (3:1 ratio of $V_{max}$ for FAXX:PAXX), whereas FAE-II demonstrated virtually no activity toward PAXX (564:1). Neither feruloyl esterase demonstrated activity toward *p*-nitrophenyl derivatives of xylose or arabinose, and both esterases hydrolyzed methyl ferulate at low rates.

TABLE 2. Substrate specificity of feruloyl esterases from *Neocallimastix* strain MC-2[a]

| Substrate | Sp act (U mg$^{-1}$) | |
|---|---|---|
| | FAE-I | FAE-II |
| FAXX[b] | 2.89 | 11.28 |
| $FAX_3$-I[b] | 3.49 | 13.87 |
| $FAX_3$-II[b] | 2.38 | 9.47 |
| PAXX[c] | 0.94 | 0.02 |
| CBG cell walls (80 μm) | 0.12 | 0.17 |
| CBG cell walls (1 mm) | 0 | 0 |
| Methyl ferulate | 0.21 | 0.002 |
| Acetylxylan | 0.009 | 0 |
| PNP-acetate[d] | 0.01 | 0 |
| PNP-xylopyranoside | 0 | 0 |
| PNP-arabinofuranoside | 0 | 0 |

[a] Average of four assays in duplicate varied less than 7%.
[b] Structures are defined in Materials and Methods and are shown in Fig. 1.
[c] PAXX is analogous to FAXX, with the *p*-coumaroyl group replacing the feruloyl group.
[d] PNP, *p*-nitrophenyl.

The feruloyl esterases had little activity toward finely ground (80 μm) CBG cell walls, releasing feruloyl groups at a rate of only 4.2 and 1.5% compared with that of FAXX for FAE-I and FAE-II, respectively; no ferulic acid was detected after hydrolysis of more coarsely ground (1 mm) CBG cell walls. These results provide further evidence that substrate size and/or accessibility impedes enzyme activity as we noticed with *p*-coumaroyl esterase (7). It has been shown that some feruloyl esterases have greater specificity for short xylo-oligomers and that a synergism occurs between xylanase (10, 22) and/or β-xylosidase (6, 7) and feruloyl esterase in the degradation of large feruloyl-polysaccharides.

The high levels of both feruloyl and *p*-coumaroyl esterases (7) present in *Neocallimastix* strain MC-2 and other rumen anaerobic fungi (3, 6) may provide a unique advantage to these microorganisms for the biodegradation of phenolic-containing and phenolic cross-linked arabinoxylans. Recent studies (1, 17) suggest that ester linkages between phenolic moieties and arabinoxylans prevent the enzymatic hydrolysis and limit utilization by bacteria of otherwise available carbohydrates. Therefore, possession of feruloyl and *p*-coumaroyl esterases, coupled with the penetrative ability provided by fungal rhizoids or rhizomycelia, provides a mechanism for the degradation and subsequent utilization of the phenolic ester-linked carbohydrates in plant cell walls.

## REFERENCES

1. **Akin, D. E., W. S. Borneman, L. L. Rigsby, and S. A. Martin.** Unpublished data.
2. **Akin, D. E., G. L. R. Gordon, and J. P. Hogan.** 1983. Rumen bacterial and fungal degradation of *Digitaria pentzii* grown with or without sulfur. Appl. Environ. Microbiol. **46:**738–748.
3. **Borneman, W. S., and D. E. Akin.** 1990. Lignocellulose degradation by rumen fungi and bacteria: ultrastructure and cell wall degrading enzymes, p. 325–339. *In* D. E. Akin, L. G. Ljungdahl, J. R. Wilson, and P. J. Harris (ed.), Microbial and plant opportunities to improve lignocellulose utilization by ruminants. Elsevier Science Publishing Co., Inc., New York.
4. **Borneman, W. S., D. E. Akin, and L. G. Ljungdahl.** 1989. Fermentation products and plant cell wall-degrading enzymes produced by monocentric and polycentric anaerobic ruminal fungi. Appl. Environ. Microbiol. **55:**1066–1073.
5. **Borneman, W. S., R. D. Hartley, D. S. Himmelsbach, and L. G. Ljungdahl.** 1990. Assay for *trans-p*-coumaroyl esterase using a specific substrate from plant cell walls. Anal. Biochem. **190:** 129–133.
6. **Borneman, W. S., R. D. Hartley, W. H. Morrison, D. E. Akin, and L. G. Ljungdahl.** 1990. Feruloyl and *p*-coumaroyl esterase from anaerobic fungi in relation to plant cell wall degradation.

Appl. Microbiol. Biotechnol. **33:**345–351.
7. **Borneman, W. S., L. G. Ljungdahl, R. D. Hartley, and D. E. Akin.** 1991. Isolation and characterization of *p*-coumaroyl esterase from the anaerobic fungus *Neocallimastix* strain MC-2. Appl. Environ. Microbiol. **57:**2337–2344.
8. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
9. **Chesson, A., and C. W. Forsberg.** 1988. Polysaccharide degradation by rumen microorganisms, p. 251–284. *In* P. N. Hobson (ed.), The rumen microbial ecosystem. Elsevier Applied Sciences, New York.
10. **Faulds, C. B., and G. Williamson.** 1991. The purification and characterization of 4-hydroxy-3-methoxycinnamic (ferulic) acid esterase from *Streptomyces olivochromogenes*. J. Gen. Microbiol. **137:**2339–2345.
11. **Ford, C. W., and R. Elliot.** 1987. Biodegradability of mature grass cell walls in relation to chemical composition and rumen microbial activity. J. Agric. Sci. Cambridge **108:**201–209.
12. **Forsberg, C. W., and K.-J. Cheng.** 1990. Integration of rumen microorganisms and their hydrolytic enzymes during digestion of lignocellulosic materials, p. 411–423. *In* D. E. Akin, L. G. Ljungdahl, J. R. Wilson, and P. J. Harris (ed.), Microbial and plant opportunities to improve lignocellulose utilization by ruminants. Elsevier Science Publishing Co., Inc., New York.
13. **Hartley, R. D., W. S. Borneman, D. S. Himmelsbach, and F. Mellon.** Unpublished data.
14. **Hartley, R. D., and C. W. Ford.** 1989. Phenolic constituents of plant cell walls and wall biodegradability, p. 137–145. *In* N. G. Lewis and M. G. Paice (ed.), Plant cell wall polymers: biogenesis and biodegradation. American Chemical Society, Washington, D.C.
15. **Hartley, R. D., and E. C. Jones.** 1976. Diferulic acid as a component of cell walls of *Lolium multiflorum*. Phytochemistry **15:**1157–1160.
16. **Hartley, R. D., W. H. Morrison, D. S. Himmelsbach, and W. S. Borneman.** 1990. Cross-linking of cell wall arabinoxylans in graminaceous plants. Phytochemistry **12:**3705–3709.
17. **Hespell, R. B., and P. J. O'Bryan.** 1992. Purification and characterization of an α-L-arabinofuranosidase from *Butyrivibrio fibrisolvens* GS113. Appl. Environ. Microbiol. **58:**1082–1088.
18. **Ishii, T.** 1991. Isolation and characterization of a diferuloyl arabinoxylan hexasaccharide from bamboo shoot cell-walls. Carbohydr. Res. **219:**15–22.
19. **Johnson, K. G., J. D. Fontana, and C. R. MacKenzie.** 1988. Measurement of acetylxylan esterase in *Streptomyces*. Methods Enzymol. **160:**551–560.
20. **Johnson, K. G., B. A. Harrison, H. Schneider, C. R. MacKenzie, and J. D. Fontana.** 1988. Xylan-hydrolysing enzymes from *Streptomyces* spp. Enzyme Microb. Technol. **10:**403–409.
21. **Jung, H. G.** 1989. Forage lignins and their effect on fiber digestibility. Agron. J. **81:**33–38.
22. **MacKenzie, C. R., and D. Bilous.** 1988. Ferulic acid esterase from *Schizophyllum commune*. Appl. Environ. Microbiol. **54:** 1170–1173.
23. **Scalbert, A., B. Monties, J.-Y. Lallemand, E. Guittet, and C. Rolando.** 1985. Ether linkage between phenolic acids and lignin fractions from wheat straw. Phytochemistry **24:**1359–1362.