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# Improving the nutritional value of oat hulls for ruminant animals with pretreatment of a multienzyme cocktail: In vitro studies<sup>1</sup>

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**ABSTRACT:** Relatively high amounts of hydroxycinnamic acid in oat hulls, mainly ferulic acid, are believed to be inhibitory to digestion by ruminal microorganisms. Ferulic acid is produced via the phenylpropanoid biosynthetic pathway and covalently cross-linked to polysaccharides by ester bonds and to components of lignin, mainly by ether bonds. Ferulic acid also forms dimers or trimers. As a result, polysaccharides become extensively cross-linked by ferulate dimerization or trimerization and incorporation into lignin. Previous studies have shown that *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase act synergistically to release ferulic acid from feruloyl-polysaccharides in complex plant cell walls of oat hulls. This activity opens the remainder of the polysaccharides to further hydrolytic attack and facilitates the accessibility of the main polysaccharide chain to cellulase, thereby increasing the release of reducing sugars. In Exp. 1, the best multienzyme cocktail (ferulic acid esterase, xylanase, cellulase, endo-glucanase [I, II], and  $\beta$ -glucanase) was developed

using an orthogonal experimental design,  $L_{25}$  ( $5^6$ ), where L = orthogonal table; 6 = factors; 5 = five levels of each; and 25 = experimental number, for further in situ and/or in vivo study. In Exp. 2, in vitro biodegradation studies with a  $3 \times 2 \times 4$  factorial arrangement of treatments were used to evaluate the responses of three feedstuffs, oat hulls or standard references (wheat straw and alfalfa hay), two particle sizes (1 mm and 250  $\mu\text{m}$ ), and four in vitro incubation treatments with the best multienzyme cocktail developed in Exp. 1. Addition of the multienzyme cocktail to the forages improved ( $P < 0.01$ ) in vitro ruminal fluid degradability. With respect to feedstuff, the order of response ( $P < 0.05$ ) to the treatments was oat hulls (+12% unit) > wheat straw (+5% unit) > alfalfa (+2% unit). This multienzyme cocktail seems best suited for oat hulls containing feruloyl ester bonds. In conclusion, data from this study suggest that the addition of the multienzyme cocktail to poorly digestible feeds before feeding enhanced degradation of DM.

Key Words: Complex Plant Cell Wall, Ferulic Acid Esterase, Feruloyl-Polysaccharides, Multienzymes, Ruminants

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## Introduction

Oat hulls contain hydroxycinnamic acids, mainly ferulic (3-methoxy-4-hydroxycinnamic acid) and *p*-coumaric acid (4-hydroxy-cinnamic acid; Yu et al., 2000, 2002a). Ferulic acid is among the factors most inhibi-

tory to the biodegradability of plant cell wall polysaccharides (Borneman et al., 1986, 1990). In monocots, ferulic acid is esterified to the C-5 hydroxyl group of some arabinopyranose residues of arabinoxylans. In dicots, ferulic acid is esterified to the C-2 hydroxyl group of arabinofuranose or to the C-6 hydroxyl group of galactopyranose residues of the pectic side chains (Brézillon et al., 1996). Ferulic acid may act as a cross-linking agent between lignin and carbohydrates or between carbohydrates (Eraso and Hartley, 1990; Iiyama et al., 1994; Bartolomé et al., 1997). These cross-linkages lead to dramatic changes in mechanical properties (Kamizaka et al., 1990; Grabber et al., 2000; Kroon et al., 1999). It also has been reported that these cross-linkages interfere with the attachment of ruminal bacteria to the plant cell wall (Borneman et al., 1986; Varel and Jung, 1986; Ishii, 1997), and as a consequence, significantly limit digestion of complex plant cell wall

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**Table 1.** Chemical composition and total extractable ferulic acid, *p*-coumaric acid, and reducing sugars of oat hulls, alfalfa, and wheat straw<sup>a</sup>

Item	Oat hulls	Alfalfa	Wheat straw
DM, g/kg	962	932	935
Ash, g/kg DM	52	98	81
CP, g/kg DM	44	189	42
ADF, g/kg DM	405	310	501
NDF, g/kg DM	775	494	740
ADL, g/kg DM	56	100	77
Hemicellulose, g/kg DM	370	184	239
Cellulose, g/kg DM	349	210	424
NDIN, g/kg CP <sup>b</sup>	191	57	70
ADIN, g/kg CP	61	13	47
Soluble CP, g/kg CP	346	536	—
Nonprotein nitrogen, g/kg CP	172	460	—
Total alkali-extractable hydrocinnamic acids, µg/mg of DM			
<i>p</i> -Coumaric acid	5.21	—	—
Ferulic acid	3.83	—	—
Total acid-extractable reducing sugar, µg/mg of DM			
Reducing sugars	793.8	—	—

<sup>a</sup>ADF and NDF are expressed as ash-inclusive. Total alkali-extractable ferulic acid, *p*-coumaric acid, and reducing sugars as reported by Yu et al. (2002a,b, 2004). The other chemical components were analyzed by Thompson et al. (2000).

<sup>b</sup>NDIN = neutral detergent insoluble N.

materials by ruminants (Borneman et al., 1986; Jung et al., 1991; Iiyama and Lam, 2001). Our hypothesis was that the release of reducing sugars and ferulic acid (disruption of cross links) from the complex cell wall of oat hulls would result in higher ruminal digestibility.

The objectives of Exp. 1 were to determine enzymatic DM disappearance of oat hulls containing feruloyl ester bonds using ferulic acid esterase in combination with other cell wall-degrading enzymes, including xylanase, cellulase, and glucanases, to obtain the best multienzyme cocktail for further in situ and/or in vitro study. The objective of Exp. 2 was to determine the effect of the multienzyme cocktail on DM disappearance of oat hulls, wheat straw (standard), and alfalfa hay (standard). Dry matter digestibility was used as an indicator of cell wall digestibility because oat hull NDF content is comparable to its DM content.

## Materials and Methods

### Oat Hulls, Wheat Straw, and Alfalfa Hay (Reference Standards) and Particle Size

Oat hulls were obtained from Can-Oat Milling Ltd. (Saskatoon, Saskatchewan, Canada). Oat hulls were screened to remove all foreign materials, and then ground to pass 1-mm and 250-µm pore-size mesh screens (Retsch ZM-1, Brinkmann Instruments Ltd., Ontario, Canada). The particle size used in this study was based on previous studies in our laboratory (Yu et al., 2002a,b; 2003). The chemical composition of oat hulls is presented in Table 1. The detailed procedures for analysis were reported by Thompson et al. (2000) and Yu et al. (2002a,b, 2003). Briefly, total alkali-extractable hydroxycinnamic acid content of oat hulls (10

mg) was determined by adding 1 M NaOH solution (0.55 mL), followed by incubation at 37°C for 24 h. After centrifugation (13,000 × *g*, 15 min), the supernatant fraction was collected, acidified with glacial acetic acid to pH 3, and extracted five times with equal volumes of ethyl acetate. The organic solutions were combined and evaporated to dryness in an evaporator unit under N<sub>2</sub>. The residue was dissolved in 1 mL of methanol/water (50:50, vol/vol) filtered through a 0.45-µm filter, and 10-µL samples were analyzed by HPLC. Total alkali-extractable ferulic and *p*-coumaric acids of oat hulls were reported previously by Yu et al. (2002a), and were 3.83 (±0.69) and 5.21 (±0.66) µg/mg (DM basis), respectively.

Total acid-extractable reducing sugars were 793.8 (±8.0) µg/mg (Yu et al., 2003). The other chemical components were analyzed by Thompson et al. (2000). Wheat straw and alfalfa hay as standard references were ground to pass through 1-mm and 250-µm pore-size mesh screens in a centrifuge mill (Retsch ZM-1).

### Enzymes and Activity Assays

*Aspergillus* ferulic acid esterase (lot No. 99021904), *Trichoderma* xylanase (lot No. 990215-04), cellulase (lot No. 99021901), endo-glucanase I (lot No. 99021902), endo-glucanase II (lot No. 99021903), and β-glucanase (lot No. BGL-098) were obtained from Finnfeeds Int. (Marlborough, U.K.). The ferulic acid esterase activity was determined by measuring the rate of hydrolysis of methyl ferulate (methyl-4-hydroxy-3-methoxy cinnamate; Apin Chemicals Ltd., Abingdon, U.K.) by HPLC using the modified methods of Faulds and Williamson (1995) and Kroon and Williamson (1996). The enzyme hydrolyses were carried out in a 100 mM 3-[*N*-morpho-

**Table 2.** Substrates and sugar standards used in enzyme activity assays

Enzyme	Substrate	Standard	Enzyme activity
<i>Aspergillus</i> ferulic acid esterase	Methyl ferulate <sup>a</sup>	Ferulic acid	46,784 $\mu\text{mol}/(\text{min}\cdot\text{mL})$ (CV < 1.0%)
Xylanase, wt/vol, %	Oat spelt xylan (wt/vol), %	D (+) xylose	579,369 $\mu\text{mol}/(\text{min}\cdot\text{g})$ (CV < 2.0%)
Cellulase, vol/vol, 1/10	Carboxymethylcellulose (wt/vol), %	D (+) glucose	27,727 $\mu\text{mol}/(\text{min}\cdot\text{mL})$ (CV < 6.4%)
$\beta$ -glucanase, vol/vol, 1/10	Barley $\beta$ -glucan (wt/vol), 0.5%	D (+) glucose	4,807 $\mu\text{mol}/(\text{min}\cdot\text{mL})$ (CV < 11.6%)
Endo-glucanase I, vol/vol, 1/10	Barley $\beta$ -glucan (wt/vol), 0.5%	D (+) glucose	6,573 $\mu\text{mol}/(\text{min}\cdot\text{mL})$ (CV < 6.8%)
Endo-glucanase II, vol/vol, 1/10	Barley $\beta$ -glucan, (wt/vol), 0.5%	D (+) glucose	6,711 $\mu\text{mol}/(\text{min}\cdot\text{mL})$ (CV < 12.3%)

<sup>a</sup>Methyl ferulate = methyl-4-hydroxy-3-methoxy cinnamate.

lino]-propane-sulfuric acid buffer at pH 6.0 in a thermostatically controlled shaking incubator at 37°C (Yu et al., 2002a). One unit of the ferulic acid esterase activity was defined as the amount of enzyme releasing 1  $\mu\text{mol}$  of ferulic acid/min. The activity assays of xylanase, cellulose, endo-glucanase (I and II), and  $\beta$ -glucanase have been reported previously (Yu et al., 2002a,b, 2003, 2004). One unit of enzyme activity (xylanase, cellulose, endo-glucanase [I and II], and  $\beta$ -glucanase) was defined as the amount of enzyme releasing 1  $\mu\text{mol}$  of sugar/min.

All assays were performed in quadruplicate and replicated four times, with blanks to correct for background in enzyme and substrate samples. The activities of the ferulic acid esterase, xylanase, cellulase, endo-glucanase I and II, and  $\beta$ -glucanase are presented in Table 2.

### Experiment 1

**Enzymes and Levels.** The experimental enzyme levels for ferulic acid esterase, xylanase, cellulase, endo-glucanase I and II, and  $\beta$ -glucanase, chosen according to previous studies (Yu et al., 2002a,b, 2003), are presented in Table 3.

*Orthogonal Experimental Design, L<sub>25</sub> (5<sup>6</sup>).* This experiment had six enzymes, each at five levels. To screen and develop the best multienzyme cocktail, an orthogonal experimental design, L<sub>25</sub> (5<sup>6</sup>), was used as shown in Table 4, where L = orthogonal table, 6 = factors, 5 = five levels of each, and 25 = experimental number. The orthogonal experimental design was based on results of previous studies (Yu et al., 2002a,b, 2003).

*Enzymatic Disappearance of Oat Hulls.* Enzymatic DM disappearance of oat hulls by each multienzyme cocktail (n = 25) was determined according to the method of Gwayumba (1997). Oat hulls were weighed (2-g original sample) into in vitro test tubes, and then treated with different enzyme combinations. Each multienzyme combination was mixed with 10 mL of sodium acetate (NaAc) buffer (pH 4.8) and applied to the substrate (5 mL of solution/g of sample). Control samples were treated with only 10 mL of NaAc buffer. All samples were incubated (40°C) in a water bath overnight and then filtered through a preweighed Whatman filter (paper No. 54, Whatman Ltd., Maidstone, U.K.) under low suction and rinsed (50 mL of distilled deionized water) to remove all solubilized material from the sam-

**Table 3.** Levels and activities of ferulic acid esterase, xylanase, cellulase, endo-glucanase I and II, and  $\beta$ -glucanase for study on digestion of oat hulls<sup>a,b</sup>

Enzymes	Activities	Dose level, U/g				
		1	2	3	4	5
<i>Aspergillus</i> ferulic acid esterase	46,784 U/mL	13 mU	260 mU	800 mU	25.6 U	409.6 U
Xylanase	579,369 U/g	7.8 mU	1 U	256 U	2,048 U	4,096 U
Cellulase	27,727 U/mL	62.5 mU	2 U	16 U	128 U	1,024 U
Endo-glucanase I	6,573 U/mL	1 U	2 U	8 U	16 U	256 U
Endo-glucanase II	6,711 U/mL	31.3 mU	250 mU	2 U	16 U	256 U
$\beta$ -glucanase	4,807 U/mL	62.5 mU	250 mU	2 U	4 U	64 U

<sup>a</sup>The ferulic acid esterase hydrolyses were carried out in a 100 mM 3-[N-morpholino]-propane-sulfuric acid buffer at pH 6.0 in a thermostatically controlled shaking incubator at 37°C (Yu et al., 2002a,b). One unit of *Aspergillus* ferulic acid esterase activity was defined as the amount of enzyme releasing 1  $\mu\text{mol}$  of ferulic acid/min.

<sup>b</sup>The enzyme activity was estimated by measuring the release of reducing sugars in a 50 mM NaAc buffer at 37°C and pH 4.8 (Yu et al., 2002a,b). One unit of the other enzyme activity was defined as the amount of enzyme releasing 1  $\mu\text{mol}$  of sugar/min (Yu et al., 2002a,b).

**Table 4.** Orthogonal experimental design, L<sub>25</sub> (5<sup>6</sup>): Effect of multienzyme cocktails on enzymatic DM disappearance of oat hulls

Exp. No.	Orthogonal experimental design, L <sub>25</sub> (5 <sup>6</sup> )						Enzymic DM disappearance, % (±SD)	Increase, % <sup>b</sup>
	Multienzyme combination level <sup>a</sup>							
	FAE level	XYL level	CEL level	EG-I level	EG-II level	β-GLU level		
Control	0	0	0	0	0	0	8.7 (± 0.3) <sup>m</sup>	—
1	1	1	1	1	1	1	9.8 (± 0.8) <sup>l</sup>	11.8
2	1	2	2	2	2	2	9.4 (± 0.1) <sup>l</sup>	7.7
3	1	3	3	3	3	3	10.1 (± 0.6) <sup>l</sup>	15.9
4	1	4	4	4	4	4	12.1 (± 0.8) <sup>ghj</sup>	39.6
5	1	5	5	5	5	5	16.3 (± 0.2) <sup>c</sup>	86.3
6	2	1	2	3	4	5	10.0 (± 0.5) <sup>l</sup>	14.3
7	2	2	3	4	5	1	11.3 (± 0.3) <sup>jk</sup>	29.7
8	2	3	4	5	1	2	12.6 (± 0.5) <sup>efgh</sup>	44.6
9	2	4	5	1	2	3	12.4 (± 0.3) <sup>fgh</sup>	42.6
10	2	5	1	2	3	4	11.4 (± 0.3) <sup>jk</sup>	30.1
11	3	1	3	5	2	4	11.7 (± 0.4) <sup>hijk</sup>	33.8
12	3	2	4	1	3	5	13.4 (± 0.9) <sup>e</sup>	53.7
13	3	3	5	2	4	1	12.3 (± 0.1) <sup>fghi</sup>	41.2
14	3	4	1	3	5	2	11.4 (± 0.3) <sup>ijk</sup>	31.1
15	3	5	2	4	1	3	11.4 (± 0.3) <sup>jk</sup>	30.1
16	4	1	4	2	5	3	12.4 (± 0.4) <sup>fgh</sup>	42.4
17	4	2	5	3	1	4	13.2 (± 0.2) <sup>ef</sup>	51.1
18	4	3	1	4	2	5	13.0 (± 0.5) <sup>efg</sup>	48.5
19	4	4	2	5	3	1	11.7 (± 0.8) <sup>hijk</sup>	34.1
20	4	5	3	1	4	2	10.9 (± 0.2) <sup>k</sup>	24.5
21	5	1	5	4	3	2	13.2 (± 0.2) <sup>ef</sup>	50.9
22	5	2	1	5	4	3	12.5 (± 0.1) <sup>efgh</sup>	43.1
23	5	3	2	1	5	4	12.8 (± 0.2) <sup>efg</sup>	46.5
24	5	4	3	2	1	5	14.2 (± 0.1) <sup>d</sup>	62.9
25	5	5	4	3	2	1	12.3 (± 0.2) <sup>fghi</sup>	40.7
SEM							0.22	

<sup>a</sup>Enzyme levels presented in Table 3. FAE = *Aspergillus* ferulic acid esterase; XYL = *Trichoderma* xylanase; CEL = cellulase; EG-I = endo-glucanase I; EG-II = endo-glucanase II; β-GLU = β-glucanase.

<sup>b</sup>Increase, % = 100 × (enzymic DM disappearance – Control)/Control.

<sup>c,d,e,f,g,h,i,j,k,l,m</sup>Means without a common superscript letter in the same column differ,  $P < 0.05$ . Main and interactive effects were all significant,  $P < 0.01$ .

ple. The residue (undigested matter) was dried overnight at 105°C and weighed. Enzyme effect was calculated as the difference in DM disappearance between the treated and the control samples (expressed as a percentage of original sample DM). All samples were determined in quadruplicate and repeated four times.

*Enzymatic Disappearance of Wheat Straw and Alfalfa Hay (Standard Samples).* To determine the value of the best multienzyme cocktail developed in the above experiment for digesting feed sources other than oat hulls, a second set of incubations was run with wheat straw and alfalfa hay (1 mm) as standard substrates. The wheat straw and alfalfa hay samples used for this trial were obtained from commercial sources through the University of Saskatchewan farm. These two feed sources were chosen because they represent a diverse range of cell wall chemical makeups and in degradability by ruminal microorganisms. The response of wheat straw and alfalfa hay to the best multienzyme cocktail was evaluated in terms of enzymatic disappearance of DM as described as above.

## Experiment 2

In vitro studies with a 3 × 2 × 4 factorial arrangement with two replicates evaluated the responses of three feedstuffs (oat hulls, wheat straw, and alfalfa hay), two particle sizes (1 mm and 250 μm), and four in vitro incubation treatments to the multienzyme cocktail developed from the previous study. The multienzyme cocktail predominantly contained ferulic acid esterase (13 mU/assay), xylanase (4,096 U/assay), cellulase (1,024 U/assay), endo-glucanase I and II (256 U/assay), and (β-glucanase (64 U/assay). The four treatments included 1) 24-h incubation in NaAc buffer (**BUFFER**); 2) 24-h incubation with the multienzyme cocktail (**ENZYME**); 3) 48-h in vitro incubation in ruminal fluid (**RUMINAL FLUID**); and 4) 24-h incubation with the multienzyme cocktail followed by a 48-h in vitro incubation in ruminal fluid (**COMBINATION**).

*In Vitro Degradation Procedure.* Each feed sample was weighed (1 g) into an in vitro tube and then mixed with 5 mL of buffer (pH 4.8), which contained the multienzyme cocktail. Control samples were treated with

**Table 5.** Composition of the supplement concentrate pellets<sup>a</sup>

Ingredient	% of DM
Barley (coarse processed)	55.77
Soybean meal, 48% CP	11.65
Canola meal	10.29
Oats	5.00
Wheat	4.09
Wheat distillers dried grains	3.26
Mineral-vitamin mix <sup>b</sup>	3.00
Corn gluten meal	2.19
Molasses	2.08
Canola oil	0.66
Cobalt-iodized salt	0.60
Sodium bicarbonate	0.60
Golden flakes <sup>c</sup>	0.45
Niacin-magnesium mix <sup>d</sup>	0.05
Ground limestone	0.05
Dynamate <sup>e</sup>	0.25

<sup>a</sup>0.48-cm pellets.

<sup>b</sup>Contained (per kilogram of concentrate) 45 mg of Mn, 63 mg of Zn, 17 mg of Cu, 0.5 mg of Se, 11,000 IU of vitamin A, 1,800 IU of vitamin D<sub>3</sub>, and 30 IU of vitamin E. The mix also contributed 0.14% Mg, 0.48% Ca, 0.26% P, 0.23% Na, and 0.38% Cl to the total concentrate (prepared by Federated Cooperatives Ltd., Saskatoon, Canada).

<sup>c</sup>Dried fat supplement distributed in Western Canada by Prairie Micro-Tech Inc., Regina, Canada.

<sup>d</sup>Contained 1 g of niacin and 0.3 g of Mg/kg of concentrate.

<sup>e</sup>Contained 22% S, 18% K, and 11% Mg (Int. Minerals and Chemicals Corp., Mundelein, IL).

only 5 mL of buffer (no multienzyme cocktail). All samples were incubated (39°C) in a water bath for 24 h and then filtered through preweighed crucibles (with Hyflo super-cel [Johns-Manville Canada Inc., Etobicoke, Ontario, Canada] and filter paper) under low suction and rinsed (50 mL of distilled deionized water) to remove all solubilized material from the samples. The residue (undigested material) was dried overnight at 105°C and weighed for DM disappearance determination.

*In Vitro Ruminant Degradation.* In vitro ruminal incubation was conducted as described for the first stage of the Tilley and Terry procedure (Marten and Barnes, 1980). Ruminant fluid was collected 1 h after the morning feeding (0800) from two ruminally cannulated, nonlactating dairy cows located at the University Dairy Experimental Station (University of Saskatchewan, Canada). Each cow received daily 15 kg (as fed) of a total mixed ration, consisting of 27.5% pelleted concentrate (as shown in Table 5), 55% barley silage, 12.5% alfalfa hay, and 5% dehydrated pelleted alfalfa (as fed) according to dairy cow maintenance requirements (NRC, 2001). Both cows were individually fed twice daily at 0800 and 1600. Water was always available. The animals used in these experiments were cared for in accordance with the guidelines of the CCAC (1993). Inocula comprising 10 mL of ruminal fluid and 15 mL of McDougall's buffer solution (Troelsen, 1966, 1971) were incubated for 48 h in the 90-mL in vitro polyethylene tubes placed in a 39°C shaking water bath. Four empty tubes also were incubated to serve as blanks.

After incubation, the residues were filtered through preweighed crucibles (crucible + Hyflo super-cel + filter paper) under low suction and rinsed (50 mL of distilled deionized water) to remove all solubilized material from the sample. The residue (undigested material) was dried overnight at 105°C and weighed for determination of DM disappearance.

*In Vitro Ruminant Degradation of Preenzyme-Treated Feedstuffs.* Each feed sample was weighed (1 g) into in vitro tubes then mixed with 5 mL of NaAc buffer (pH 4.8), which contained the multienzyme cocktail. Control samples were treated with only 5 mL of NaAc buffer (no multienzyme cocktail). All samples were incubated (39°C) in a water bath for 24 h and freeze dried. The preenzyme-treated samples were then added to a mixture of ruminal fluid and McDougall's buffer solution for 48 h (Marten and Barnes, 1980). The ruminal fluid and McDougall's buffer solution used were the same as stated above (Troelsen, 1966, 1971). After incubation, the residues were filtered through preweighed crucibles (with Hyflo super-cel and filter paper) under low suction and rinsed (50 mL of distilled deionized water) to remove all solubilized material from the sample. The residue (undigested material) was dried overnight at 105°C and weighed to determine DM disappearance.

### Statistical Analyses

Statistical analysis was carried out using SAS (SAS Inst., Inc., Cary, NC). The data from Exp. 1 were analyzed as a completely randomized design using the Proc GLM procedure, with a model including main effects (each enzyme) and enzyme interactions. Treatment means were compared using the Student-Newman-Keuls test (Steel and Torrie, 1980). Significance was declared at  $P < 0.05$ . The data from Exp. 1 also were analyzed by a stepwise regression using Proc REG of SAS.

Data from Exp. 2 were analyzed as a  $3 \times 2 \times 4$  factorial arrangement in a completely randomized design with the following model: DM disappearance = mean + forage + particle size + treatment + forage  $\times$  particle size + forage  $\times$  treatment + particle size  $\times$  treatment + forage  $\times$  particle size  $\times$  treatment + error. Treatment means were compared using the Student-Newman-Keuls test (Steel and Torrie, 1980), and significance was declared at  $P < 0.05$ .

### Results and Discussion

Previous studies in our laboratory have found that 1) *Aspergillus* ferulic acid esterase was able to cleave ferulic acid from the sugar moiety of the complex cell wall of oat hulls containing feruloyl ester bonds (Yu et al., 2002a, 2004); 2) efficient release of ferulic acid depended on substrate particle size ( $\leq 250 \mu\text{m}$ ) and also on the presence of the cell wall-degrading enzyme *Trichoderma* xylanase (Yu et al., 2002a,b, 2004); and 3) ferulic acid esterase, xylanase, and cellulase acted syn-

**Table 6.** Summary of a stepwise regression analysis for effect of multienzyme cocktails on enzymatic DM disappearance of oat hulls<sup>a</sup>

Step	Variable entered	Number and names of variables <sup>b</sup>	Model R <sup>2</sup> value	P-value
1	CEL	1 (CEL)	0.253	<0.001
2	$\beta$ -GLU	2 ( $\beta$ -GLU, CEL)	0.445	<0.001
3	FAE	3 (FAE, $\beta$ -GLU, CEL)	0.546	<0.001
4	EG-I	4 (EG-I, FAE, $\beta$ -GLU, CEL)	0.635	<0.001
5	EG-II	5 (EG-II, EG-I, FAE, $\beta$ -GLU, CEL)	0.700	<0.001
6	XYL	6 (XYL, EG-II, EG-I, FAE, $\beta$ -GLU, CEL)	0.737	<0.001

<sup>a</sup>Enzyme levels presented in Table 3. FAE = *Aspergillus* ferulic acid esterase; XYL = *Trichoderma* xylanase; CEL = cellulase; EG-I = endo-glucanase I; EG-II = endo-glucanase II;  $\beta$ -GLU =  $\beta$ -glucanase.

<sup>b</sup>Variables included in the stepwise regression analysis.

ergistically to efficiently release reducing sugars from the complex cell walls of oat hulls (Yu et al., 2003). It was postulated that the synergistic interaction between ferulic acid esterase and xylanase results in breaking the ester linkage between ferulic acid and the attached sugar of feruloyl-polysaccharides of the cell wall, making the cell wall structure more available to attack by the cellulase. This action resulted in a greater release of reducing sugars.

### Experiment 1

*Effect of Multienzyme Cocktail on Disappearance of Oat Hulls.* The effects of the ferulic acid esterase in combination with other cell wall-degrading enzymes (xylanase, cellulase, and glucanases) on enzymatic DM disappearance of oat hulls are presented in Table 4. All combinations of cell wall-degrading enzymes increased ( $P < 0.01$ ) enzymatic DM disappearance by 7.7 to 86.3% compared with the control. The greatest enzymatic DM disappearance (16.3%) was found at 13 mU of ferulic acid esterase; 4,096 U of xylanase; 1,024 U of cellulase; 256 U of endo-glucanase I and II; and 64 U of  $\beta$ -glucanase. This multienzyme cocktail contained the lowest level of ferulic acid esterase and the highest levels of the other cell wall-degrading enzymes. The results from the stepwise regression analysis showed that all enzyme activities could explain 74% of the variation in enzymatic DM disappearance, and the three enzyme activities (ferulic acid esterase,  $\beta$ -glucanase, and cellulase) could explain 55% of the variation in enzymatic DM disappearance (Table 6). The previous studies showed that ferulic acid esterase together with the other cell wall-degrading enzymes could synergistically act to disrupt hydroxycinnamic cross-linked complex cell wall polysaccharides to efficiently release ferulic acid and reducing sugars from the oat hulls (Yu et al., 2002a,b, 2003). This may be the explanation for the observed increase in enzymatic DM disappearance of oat hulls. Such pretreatment may provide a unique advantage to ruminal microorganisms for the digestion of the ferulic cross-linked complex cell wall of oat hulls. Multienzymatic pretreatment may be a nutritional strategy that can be used to improve the digestibility

and utilization of oat hulls and other high-fiber plant materials.

*Effect of the Multienzyme Cocktail on the Disappearance of Alfalfa Hay and Wheat Straw (Standard Laboratory Samples).* The effects of the best multienzyme cocktail (ferulic acid esterase, xylanase, cellulase, endo-glucanase I and II, and  $\beta$ -glucanase) on enzymatic DM disappearance of alfalfa hay and wheat straw (1 mm) are presented in Table 7. The best multienzyme cocktail developed in Exp. 1 increased ( $P < 0.05$ ) enzymatic DM disappearance for both alfalfa hay and wheat straw compared with the control. Responses to the multienzyme cocktail between the two types of feedstuffs were different, with a greater improvement in enzymatic disappearance for wheat straw (153.9%) than for alfalfa hay (55.6%). This result may be due to differences in their (bio)chemical structures. Wilman et al. (1999) suggested that the low digestibility of mature plant tissue, such as straws, is not due to thick vascular strands or to a high proportion of vascular tissue, but rather to a high proportion of cell wall and incomplete and delayed access by ruminal microorganisms to much of the cell wall. It seems that the multienzymatic cocktail developed in the present study is more suitable for poorly digested feedstuffs like oat hulls and wheat straw.

### Experiment 2

The main effects of substrate, particle size, and treatments on DM disappearance, as well as their two-way and three-way interactions ( $P < 0.05$ ), are presented in Table 8. The particle size of oat hulls affected the magnitude of response to pretreatment with the cocktail. Pretreatment of oat hulls with the multienzyme cocktail increased ( $P < 0.05$ ) DM disappearance in ruminal fluid. With a particle size of 1 mm, DM disappearance increased by 6.5 percentage units; however, with a particle size of 250  $\mu$ m, DM disappearance increased by 12.1 percentage units. The ability of the ENZYME treatment to degrade oat hulls with particle size of 1 mm was 10 percentage units lower than that by the RUMINAL FLUID treatment. With a particle size of 250  $\mu$ m, the ability of ENZYME treatment to degrade oat hulls was improved, but DM disappearance was

**Table 7.** Effect of the multienzyme cocktail on enzymatic dry matter disappearance of alfalfa hay and wheat straw with particle size of 1 mm<sup>a</sup>

	Multienzyme cocktail						Experimental results		
	FAE level	XYL level	CEL level	EG-I level	EG-II level	$\beta$ -GLU level	Enzymic DM disappearance, % ( $\pm$ SD)	Enzymic effect <sup>b</sup>	Increase, % <sup>c</sup>
Alfalfa hay									
Control <sup>d</sup>	0	0	0	0	0	0	24.6 ( $\pm$ 0.64) <sup>f</sup>	—	—
Enzyme	1	5	5	5	5	5	38.2 ( $\pm$ 0.82) <sup>e</sup>	13.7	55.6
Wheat straw									
Control	0	0	0	0	0	0	9.4 ( $\pm$ 0.05) <sup>g</sup>	—	—
Enzyme	1	5	5	5	5	5	23.8 ( $\pm$ 1.06) <sup>f</sup>	14.4	153.9
SEM							0.52		

<sup>a</sup>FAE = *Aspergillus* ferulic acid esterase; XYL = *Trichoderma* xylanase; CEL = cellulase; EG-I = endo-glucanase I; EG-II = endo-glucanase II;  $\beta$ -GLU =  $\beta$ -glucanase.

<sup>b</sup>Enzymic effect (increase % units) = Enzymic DM disappearance – Control.

<sup>c</sup>Increase, % =  $100 \times (\text{enzymic DM disappearance} - \text{Control})/\text{Control}$ .

<sup>d</sup>Control were treated with only NaAc buffer.

<sup>e,f,g</sup>Means without a common superscript letter in the same column differ,  $P < 0.05$ .

only four percentage units different than the RUMINAL FLUID treatment. The particle size of oat hulls affected the response to pretreatment of the multienzyme, which could be explained in part by our previous studies (Yu et al., 2002a,b). This multienzyme cocktail contains ferulic acid esterase. Ferulic acid esterase in combination with xylanase breaks the ester linkage between ferulic acid and the attached sugar, releasing ferulic acid from the hydroxycinnamic cross-linked complex plant cell walls of oat hulls. However, the release of ferulic acid by ferulic acid esterase depends on the particle size of oat hulls ( $\leq 250 \mu\text{m}$ ). With a particle size of 1 mm, ferulic acid esterase had little effect on the release of ferulic acid from the complex cell walls of oat hulls. An increase in the surface area available for enzymatic attack and/or enhanced accessibility to substrate could be potential reasons for increased DM disappearance with decreased particle size (Yu et al., 2002a,b).

In alfalfa hay, the pretreatment with the multienzyme cocktail did not increase DM disappearance in ruminal fluid with the particle size of 1 mm and improved little with the particle size of  $250 \mu\text{m}$ , indicating that pretreatment with the multienzyme cocktail (the best combination) did not work as well with more digestible substrate. The reason may be because alfalfa contains little *p*-coumaric and ferulic acid (Graham and Aman, 1983; Scalbert et al., 1985; Titgemeyer et al., 1991).

In wheat straw, pretreatment with the multienzyme cocktail increased ( $P < 0.05$ ) DM disappearance in ruminal fluid. With a particle size of 1 mm, DM disappearance increased 6.5 percentage units, which was similar to oat hulls. With particle size of  $250 \mu\text{m}$ , DM disappearance increased ( $P < 0.05$ ) only 5.1 percentage units, which was quite lower than that in oat hulls (12.1 percentage unit increase).

The responses of alfalfa hay (least response), wheat straw (moderate response), and oat hulls (greatest response) to the multienzyme cocktail could be due mainly to their internal plant cell wall structures (such as hydroxycinnamic acid content and lignification). For example, oat hulls contain relatively high ferulic acid (3-methoxy-4-hydroxycinnamic acid) and *p*-coumaric acid (4-hydroxycinnamic acid). These acids are covalently cross-linked to polysaccharides and to lignin components (Scalbert et al., 1985; Borneman et al., 1991), resulting in limited cell-wall degradability (Borneman et al., 1986; Hartley and Ford, 1989; Brézillon et al., 1996). It seems that the multienzyme cocktail is best suited for oat hulls, followed by wheat straw. The results indicate that addition of the multienzyme cocktail to poorly digestible feed with a relatively high amount of hydroxycinnamic acids before feeding has the potential to enhance degradation and digestion. Results of our previous studies indicate that synergistic interaction between ferulic acid esterase and xylanase to release ferulic acid from feruloyl-polysaccharides from oat hull makes the remainder of the polysaccharides vulnerable to further hydrolytic attack and facilitates the accessibility to the main chain of polysaccharide by the cell wall-degrading enzymes. This action facilitates cell wall hydrolysis, thereby releasing a higher yield of reducing sugars. In the present study, increased DM disappearance with the multienzyme pretreatment may have occurred due the actions noted in the previous work. Such multienzyme pretreatment could provide a unique advantage to ruminal microorganisms for the biodegradation of the complex plant cell walls with relatively high amount of hydroxycinnamic acids.

In conclusion, multienzyme cocktails (ferulic acid esterase in combination with the other cell wall-degrading enzymes) increased enzymatic DM disappearance of oat hulls. The greatest enzymatic DM disappearance

**Table 8.** Dry matter disappearance of oat hulls, wheat straw, and alfalfa hay

Forages	Particle size	Treatments <sup>a</sup>	DM disappearance, % (±SD)
Oat hulls	1 mm	BUFFER	7.5 (± 2.0) <sup>q</sup>
		ENZYME	11.0 (± 2.1) <sup>o</sup>
		RUMINAL FLUID	21.2 (± 1.4) <sup>kl</sup>
		COMBINATION	27.7 (± 0.3) <sup>j</sup>
	250 μm	BUFFER	10.3 (± 1.7) <sup>op</sup>
		ENZYME	18.6 (± 0.4) <sup>m</sup>
		RUMINAL FLUID	22.8 (± 0.8) <sup>kl</sup>
		COMBINATION	34.8 (± 0.7) <sup>h</sup>
Wheat straw	1 mm	BUFFER	6.8 (± 0.6) <sup>q</sup>
		ENZYME	14.5 (± 1.0) <sup>n</sup>
		RUMINAL FLUID	23.4 (± 0.8) <sup>k</sup>
		COMBINATION	29.9 (± 1.2) <sup>i</sup>
	250 μm	BUFFER	8.6 (± 0.7) <sup>pq</sup>
		ENZYME	20.5 (± 1.8) <sup>km</sup>
		RUMINAL FLUID	26.6 (± 0.9) <sup>j</sup>
		COMBINATION	31.7 (± 1.4) <sup>i</sup>
Alfalfa hay	1 mm	BUFFER	36.1 (± 1.8) <sup>h</sup>
		ENZYME	42.7 (± 0.5) <sup>f</sup>
		RUMINAL FLUID	59.0 (± 0.7) <sup>d</sup>
		COMBINATION	60.1 (± 1.0) <sup>d</sup>
	250 μm	BUFFER	38.0 (± 2.2) <sup>g</sup>
		ENZYME	45.8 (± 1.7) <sup>e</sup>
		RUMINAL FLUID	60.3 (± 2.2) <sup>d</sup>
		COMBINATION	62.6 (± 2.1) <sup>c</sup>
SEM			0.69
			— P-value —
Statistical analysis <sup>b</sup>			
Forage			<0.001
Particle size			<0.001
Treatment			<0.001
Forage × particle size			0.001
Forage × treatment			<0.001
Particle size × treatment			<0.001
Forage × particle size × treatment			0.014

<sup>a</sup>Treatments: BUFFER = 24-h incubation with NaAc buffer only; ENZYME = 24-h incubation with the multienzyme preparation; RUMINAL FLUID = 48-h in vitro incubation with ruminal fluid only; and COMBINATION = 24-h incubation with the multienzyme preparation first and then with ruminal fluid for 48-h incubation.

<sup>b</sup>The data were analyzed by a 3 × 2 × 4 factorial analysis.

<sup>c,d,e,f,g,h,i,j,k,l,m,n,o,p,q</sup>Means without a common superscript letter in the same column differ,  $P < 0.05$ .

was found at 13 mU of ferulic acid esterase, 4,096 U of xylanase, 1,024 U of cellulase, 256 U of endo-glucanase I and II, and 64 U of β-glucanase. Addition of the multienzyme cocktail to the forages improved in vitro ruminal fluid degradation. The response to the multienzyme cocktail often increased when the forage was ground to 250 μm. With respect to feedstuff, the following order of response to the multienzyme treatments was observed: oat hulls > wheat straw > alfalfa. The multienzyme cocktail seems most suitable for oat hulls, and it is likely that ruminal digestion of the hydroxycinnamic cross-linked complex cell wall of oat hulls could be improved with such multienzymatic pretreatment. The results from this study suggest that the addition of the multienzyme cocktail to poorly digestible feeds before feeding has the potential to enhance ruminal degradation and digestion.

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