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Enzymic Release of Reducing Sugars from Oat Hulls by Cellulase, as Influenced by *Aspergillus* Ferulic Acid Esterase and *Trichoderma* Xylanase

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Hydroxycinnamic acids, mainly ferulic and *p*-coumaric acids, are believed to be inhibitory to ruminal biodegradability of complex cell wall materials such as oat hulls. Previous studies have shown that a novel enzyme, *Aspergillus* ferulic acid esterase, and *Trichoderma* xylanase act synergistically to break the ester linkage between ferulic acid and the attached sugar of feruloyl polysaccharides, releasing ferulic acid from oat hulls. In this paper, we examined the enzymic release of reducing sugars from oat hulls by the actions of individual enzymes (*Aspergillus* ferulic acid esterase at 13 mU, 6.4 U, and 4678.4 U/assay; cellulase at 20 levels, ranging from 7.8 mU to 2772.7 U/assay; *Trichoderma* xylanase at 20 levels, ranging from 7.8 mU to 4096 U/assay) and by the combined action of cellulase at six levels (62.5 mU, 2 U, 16 U, 128 U, 1024 U, and 2772.7 U/assay), *Aspergillus* ferulic acid esterase at 13 mU/assay, and *Trichoderma* xylanase at two levels (1 U and 256 U/assay). The amount of total acid-extractable reducing sugars in the oat hulls used in this study was 793.8 ± 8.0 μg/mg. The results show that after a 24-h incubation with *Aspergillus* ferulic acid esterase alone, no reducing sugars were observed to be released from oat hulls. With cellulase as the sole enzyme, as the concentration increased from 7.8 mU to 2772.7 U/assay, the release of reducing sugars increased ($P < 0.01$) from 0 to 39% of the total present, with the highest release at 512 U/assay. With *Trichoderma* xylanase alone, as the concentration increased from 7.8 mU to 4096 U/assay, the release of reducing sugars increased ($P < 0.01$) from 4.9 to 33%, with the highest release at 2048 U/assay. When incubated together with *Trichoderma* xylanase (1 U or 256 U/assay) and *Aspergillus* ferulic acid esterase (13 mU/assay), cellulase at all six levels (62.5 mU, 2 U, 16 U, 128 U, 1024 U and 2772.7 U/assay) significantly increased the release of reducing sugars ($P < 0.01$) from 8 to 69%. These results indicate that the synergistic interaction between *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase on the release of ferulic acid from feruloyl polysaccharides of oat hulls makes the remainder of the polysaccharides open for further hydrolytic attack and facilitates the accessibility of the main chain of polysaccharides to cellulase. This action extends the cell wall hydrolysis, thus releasing a higher yield of reducing sugars. Such enzymic pretreatment of oat hulls may provide a unique advantage to rumen microorganisms for the biodegradation of the complex cell walls of byproduct feeds such as oat hulls.

KEYWORDS: Ferulic acid esterase; polysaccharide degrading enzyme; synergistic interaction; feruloyl polysaccharides; hydroxycinnamic acids; reducing sugars; biodegradation; biobarrier; complex plant cell walls; oat hulls

INTRODUCTION

In complex plant cell walls, ferulic acid is ubiquitous (*1*) and is found in particularly high concentration in the cell walls of cereal grains (*2*), such as oat (*3*, *4*). Ferulic acid is a hydroxycinnamic acid and is produced via the phenylpropanoid biosynthetic pathway (*5*). Ferulic acid is found covalently cross-linked to polysaccharides by ester bonds and to components of lignin mainly by ether bonds (*6*).

In monocots, ferulic acid is esterified to the C-5 hydroxyl group of some arabinopyranose residues of arabinoxylans (such as wheat bran, oat hulls, barley straw, and maize). In dicots, ferulic acid is esterified to the C-2 hydroxyl group of arabino-

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furanose or to the C-6 hydroxyl group of galactopyranose residues of the pectic side chains such as sugar beet and spinach (*1*, *7*, *8*).

Dimerization of ferulic acid side chains by oxidative coupling has been reported (*9*). Ferulic acid may act as a cross-linking agent between lignin and carbohydrates or between carbohydrates. Several studies (*2*, *10*, *11*) have shown that a proportion of the ferulic acid is present as dimers which function to cross-link and strengthen the cell wall in plants such as ryegrass, barley straw, red clover, maize, and sorghum (*2*). The examples of dimeric structures that have been identified include 5-8′BenDi {*trans*-5-[(*E*)-2-carboxyvinyl]-2-(4-hydroxy-3-methoxy-phenyl)-7-methoxy-2,3-dihydrobenzofuran-3-carboxylic acid}, 5-8′diFA {(*E,E*)-4,4′-dihydroxy-3,5′-dimethoxy-β,3′-bicinnamic acid}, and 8-O-4′diFA {(*Z*)-β-{4-[(*E*)-2-carboxyvinyl]-2-methoxyphenoxy}-4-hydroxy-3-methoxycinnamic acid}. The linkage can be through either the phenolic ring or the unsaturated aliphatic side chain (*12*, *13*).

These cross-links have a number of physical consequences, including influences on nutritional value and on cell wall growth and mechanical strength. Studies involving various methodologies have consistently indicated that ferulic acid constituents are among the factors most inhibitory to the biodegradability of plant cell wall polysaccharides (*14*, *15*). Hartley and Ford (*16*) showed that ferulic acid of cell wall materials influenced the biodegradability of cell wall polysaccharides. Graham and Aman (*17*) reported that ferulic acid restricted the digestibility of forage by ruminants. Inhibition to biodegradability of plant cell wall polysaccharides might be due to inhibition of rumen bacteria by ferulic acid (*14*) or due to limitation of rumen bacterial hydrolysis by the ferulic acid-containing and ferulic acid-cross-linked complex cell walls (*16*). Bohn and Fales (*18*) showed that hydroxycinnamic acid was esterified in vitro to neutral detergent fiber of switchgrass and, as a result, reduced digestibility dramatically. This further supports the view that esterified ferulic acid in plant cell walls is a barrier to biodegradation (*19*). Therefore, for a more complete degradation of cell wall polymers, a ferulic acid esterase(s) capable of hydrolyzing these bonds is required in addition to polysaccharide-degrading enzymes.

Recent research in our laboratory (*3*, *4*, *20*) has shown that *Aspergillus* ferulic acid esterase was able to cleave the ferulic acid from the sugar moiety and release free ferulic acid from oat hulls. The research has also shown that release of ferulic acid by *Aspergillus* ferulic acid esterase was dependent upon the particle size ($\leq$250 μm) of oat hulls and the presence of *Trichoderma* xylanase. Several other studies have also shown that efficient release of ferulic acid from plant cell walls is not only dependent on substrate particle size (*15*) but also dependent on the presence of other cell wall-degrading enzymes, such as xylanase (*7*, *21*).

This research (*7*, *15*, *21*) indicates a synergistic interaction between *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase on the release of ferulic acid of feruloyl polysaccharides of the plant cell walls. Whether this synergistic interaction between *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase could enhance the release of reducing sugars by other cell wall-degrading enzymes, such as cellulase, is in question. The objectives of this study were to determine the release of reducing sugars from oat hulls containing feruloyl ester bonds by cellulase, as influenced by the synergistic interaction (*20*) between *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase.

MATERIALS AND METHODS

Oat Hulls and Particle Size. Oat hulls were obtained from Can-Oat Milling Ltd. (Saskatchewan, Canada). Oat hulls were first screened to removed grain, small kernels, and other foreign materials and then ground through a 250-μm-pore-size mesh screen (Retsch ZM-1). The particle size used in this study was based on the previous studies in our laboratory (*4*, *20*).

Enzymes and Activity Assays. Enriched sources of *Aspergillus* ferulic acid esterase (Lot 99021904), cellulase (Lot 99021901), and *Trichoderma* xylanase (Lot 990215-04) were obtained from Finnfeeds International, UK. As previously reported, there was no detectable cross contamination (*4*) in each enzyme.

Aspergillus ferulic acid esterase activity was determined by measuring the rate of hydrolysis of methyl ferulate (MF, methyl 4-hydroxy-3-methoxycinnamate; Apin Chemicals Limited, UK) by HPLC using the modified methods of Faulds and Williamson (*7*) and Kroon and Williamson (*22*). The enzyme hydrolyses were carried out in 100 mM 3-(*N*-morpholino)propanesulfuric acid (MOPS) buffer at pH 6.0 in a thermostatically controlled shaking incubator at 37 °C, as previously described (*4*). One unit (U) of *Aspergillus* ferulic acid esterase activity was defined as the amount of enzyme releasing 1 μmol of ferulic acid per minute.

Trichoderma xylanase activity was estimated by measuring the release of reducing sugars (*23*, *24*) from 1% (w/v) soluble oat spelt xylan (Sigma, X-0627, Lot 70H094) (*25*) in a 50 mM NaAc buffer at 37 °C, pH 4.8, and expressed as xylose equivalents (Sigma-D (+), X-3877). One unit (U) of *Trichoderma* xylanase activity was defined as the amount of enzyme releasing 1 μmol of sugars per minute.

Cellulase activity was estimated by measuring the release of reducing sugars (*23*, *24*) from carboxymethylcellulose (CMC; w/v, %; Sigma, C-4888, Lot 119F0611) by cellulase (v/v, 1/10) in a 50 mM NaAc buffer at 37 °C, pH 4.8, and expressed as glucose equivalents (D-glucose, anhydrous, BDH). One unit (U) of cellulase ctivity was defined as the amount of enzyme releasing 1 μmol of sugar per minute.

All assays were performed in quadruplicate and replicated four times, with blanks to correct for background in enzyme and substrate samples. The activities of *Aspergillus* ferulic acid esterase, *Trichoderma* xylanase, and cellulase were 46 784 U/mL (coefficient of variation, <1%), 579 369 U/g (coefficient of variation, <2%), and 27 727 U/mL (coefficient of variation, <6.4%), respectively.

Total Content of Acid-Extractable Reducing Sugars. The total acid-extractable reducing sugar content was measured according to the methods of Dubois et al. (*23*), Miller (*24*), and Englyst and Hudson (*26*), with some modifications. A brief description of the procedure is as follows.

Reducing sugars were determined by the 3,5-dinitrosalicylic acid (DNSA) method by treating oat hulls (100 mg) with 12 M sulfuric acid for 1 h (35 °C and in the dark), followed by placing them in boiling water for 2 h. The soluble fraction, after centrifugation at 3000*g* for 15 minutes was neutralized with 3.9 M sodium hydroxide, and then the reducing sugar level was analyzed by a spectrophotometer (UltrospecIII, model 80-2097-62, Pharmacia LKB) by reference to glucose standards (D-glucose, anhydrous, Code B28450, BDH Chemical Ltd.). Glucose was used as the sole standard, because only small differences are seen between a mixed sugar standard and a glucose standard in color yield upon their reaction with DNSA, as reported by Englyst and Hudson (*26*). All assays were performed in quadruplicate and replicated four times.

Total Alkali-Extractable Ferulic and *p*-Coumaric Acids. The total alkali-extractable content of ferulic and *p*-coumaric acids in 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 (13000*g*, 15 min), the supernatant was collected, acidified with 200 μL of 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_2. The residue was dissolved in 1 mL of methanol/water (50:50, v/v) and filtered through a 0.45-μm filter, and then 10-μL samples were analyzed by HPLC (a Beckman chromatograph equipped with a 126 programmable solvent module, a

Table 1. Chemical Composition of Oat Hulls

chemical composition[a]	contents
total alkali-extractable hydroxycinnamic acids (μg/mg)	
p-coumaric acid	5.21
ferulic acid	3.83
total acid-extractable reducing sugar (μg/mg)	
reducing sugars	793.8
dry matter (g/kg)	961.8
ash (g/kg DM)	52.3
crude protein (g/kg DM)	44.3
acid detergent fiber (g/kg DM)	405.1
neutral detergent fiber (g/kg DM)	775.2
lignin (g/kg DM)	56.1
hemicellulose (g/kg DM)	370.0
cellulose (g/kg DM)	349.1
neutral detergent insoluble nitrogen (g/kg CP)	191.3
acid detergent insoluble nitrogen (g/kg CP)	60.8
soluble crude protein (g/kg CP)	346.1
non-protein nitrogen (g/kg CP)	172.2

[a] DM, dry matter; CP, crude protein.

507 System Gold autosampler, and a RF-551 PC spectrofluorometric detector) (*4*, *20*). Samples were prepared and analyzed in triplicate. All assays were replicated three times.

Enzymic Release of Reducing Sugars. The release of reducing sugars from the oat hulls by each enzyme or enzyme mixture was determined by the DNSA method (*23*−*25*). The concentrations of *Aspergillus* ferulic acid esterase at 13 mU/assay and *Trichoderma* xylanase at 1 and 256 U/assay were chosen in this trial on the basis of previous studies in our laboratory (*4*, *20*). Enzymatic hydrolysis was carried out in 50 mM NaAc buffer, pH 4.8, in a thermostatically controlled shaking water bath at 37 °C. Oat hulls (sample size 5 mg), preheated to 37 °C, were added promptly and mixed with 100 μL of the specific enzyme solutions, which differed in enzyme levels. The mixture was incubated for 24 h. Blanks were included for each enzyme and substrate to correct background errors. After incubation, 500 μL of dilute DNSA solution was added. Each test tube was then placed in a boiling water bath for exactly 5 min to stop the enzyme reaction, 1 mL of distilled water was added promptly to the solution, and the tubes were cooled to room temperature. The samples were then centrifuged (3000*g*, 15 min) before the color development was read at 575 nm on the spectrophotometer (UltrospecIII, model 80-2097-62, Pharmacia LKB). Reducing sugar release was interpreted by using a glucose (D-glucose, anhydrous, Code B28450, BDH Chemical Ltd.) standard curve. Each sample was prepared and analyzed in quadruplicate.

Statistical Analysis. Statistical analyses were carried out using Proc GLM from SAS (*27*). Treatment means were compared using the Student−Newman−Keuls test (*28*). Linear and quadratic effects of the enzyme(s) concentration were evaluated using Proc GLM (*27*). Significance was declared at $P < 0.05$.

RESULTS

Total Contents of Reducing Sugars, Ferulic Acid, and *p*-Coumaric Acid and Chemical Composition of Oat Hulls. The chemical composition of oat hulls is presented in **Table 1**. The amount of total acid-extractable reducing sugars was 793.8 ± 8.0 μg/mg. The amounts of total alkali-extractable ferulic and *p*-coumaric acids in the oat hulls used in this study were reported previously by Yu et al. (*4*) and were 3.83 ± 0.69 and 5.21 ± 0.66 μg/mg, respectively. The other chemical composition of oat hulls was analyzed by Thompson et al. (*29*) in our laboratory.

Release of Reducing Sugars from Oat Hulls by *Aspergillus* Ferulic Acid Esterase. The release of reducing sugars by *Aspergillus* ferulic acid esterase was examined after a 24-h incubation to check whether *Aspergillus* ferulic acid esterase alone could release any reducing sugars. The concentrations of enzyme used were 13 mU, 6.4 U, and 4678.4 U/assay, representing relatively low, medium, and high levels of *Aspergillus* ferulic acid esterase. The results showed that there was no release of reducing sugars when oat hulls were incubated with only *Aspergillus* ferulic acid esterase (data not shown).

Table 2. Effect of Cellulase at Concentrations Ranging from 7.8 mU to 2772.7 U/Assay on the Release of Reducing Sugars from Oat Hulls with a Particle Size of 250 μm after a 24-h Incubation[a]

cellulase level (mU or U/assay)	reducing sugars released (μg)[b]	SD[c]	% of total reducing sugars[b,d]	SD[c]
7.8 mU	nd[e]		nd	
15.6 mU	nd		nd	
31.3 mU	nd		nd	
62.5 mU	nd		nd	
125 mU	109.1 ij	±52.2	2.7 ij	±1.3
250 mU	46.3 j	±15.1	1.2 i	±0.4
500 mU	174.4 i	±57.3	4.4 i	±1.4
1 U	280.9 h	±44.9	7.1 h	±1.1
2 U	333.7 h	±26.5	8.4 h	±0.7
4 U	494.6 g	±54.1	12.5 g	±1.4
8 U	723.6 f	±34.3	18.2 f	±0.9
16 U	866.6 e	±46.3	21.8 e	±1.2
32 U	1222.8 d	±110.2	30.8 d	±2.8
64 U	1218.1 d	±46.2	30.7 d	±1.2
128 U	1415.0 bc	±48.8	35.7 bc	±1.2
256 U	1469.8 abc	±69.6	37.0 abc	±1.8
512 U	1533.4 a	±60.8	38.6 ab	±1.5
1024 U	1509.1 ab	±21.7	38.0 a	±0.6
2048 U	1392.3 c	±42.8	35.1 c	±1.1
2772.7 U	1465.3 abc	±57.8	36.9 abc	±1.5
SEM[f]	26.82		0.67	

Main Effect of Cellulase

	linear effect	quadratic effect
reducing sugars released (μg)	$P < 0.001$	$P < 0.001$
% of total reducing sugars	$P < 0.001$	$P < 0.001$

[a] Enzymic hydrolyses were carried out in 50 mM NaAc buffer, pH 4.8, in a thermostatically controlled shaking water bath at 37 °C for 24 h. [b] Means with the same letter in the same column are not significantly different. [c] Standard deviation. [d] 100% reducing sugar was defined as the content of total acid-extractable reducing sugars in oat hulls. [e] nd, not detectable. [f] Standard error of means.

Release of Reducing Sugars from Oat Hulls by Cellulase. The release of reducing sugars from oat hulls by the action of cellulase alone was determined at concentrations ranging from 7.8 mU to 2772.7 U/assay, after a 24-h incubation. The results are shown in **Table 2**. At relatively low levels of cellulase (7.8 to 62.5 mU/assay), no release of reducing sugars was detected. With increasing cellulase level (125 mU to 512 U/assay), the release of reducing sugars was increased from 1.2 to 38.6%, with the highest release at 512 U/assay. Further increasing the cellulase level from 1024 to 2772.7 U/assay resulted in no further increase in the release of reducing sugars.

Polynomial regression analysis showed a quadratic effect ($P < 0.001$) of cellulase concentration on the release of reducing sugars from oat hulls. The equations for estimating the release of reducing sugars are as follows:

$$\text{release of reducing sugars } (\mu\text{g}) = 623.86 + 1.55[\text{CEL}] - 0.00048[\text{CEL}]^2$$

Table 3. Effect of *Trichoderma* Xylanase at Concentrations Ranging from 7.8 mU to 4096 U/Assay on the Release of Reducing Sugars from Oat Hulls with a Particle Size of 250 μm after a 24-h Incubation[a]

xylanase level (mU or U/assay)	reducing sugars released (μg)[b]	SD[c]	% of total reducing sugars[b,d]	SD[c]
7.8 mU	193.6 i	±66.9	4.9 i	±1.7
15.6 mU	280.8 ghi	±24.2	7.1 ghi	±0.6
31.3 mU	221.1 hi	±48.1	5.6 hi	±1.2
62.5 mU	291.0 ghi	±44.5	7.3 ghi	±1.1
125 mU	349.3 fghi	±82.9	8.8 fghi	±2.1
250 mU	248.3 ghi	±19.7	6.3 ghi	±0.5
500 mU	209.7 hi	±13.0	5.3 hi	±0.3
1 U	386.5 fg	±36.6	9.7 fg	±0.9
2 U	222.2 hi	±43.0	5.6 hi	±1.1
4 U	247.4 fgh	±36.2	6.2 fgh	±0.9
8 U	265.6 ghi	±39.5	6.7 ghi	±1.0
16 U	237.1 ghi	±35.3	4.1 ghi	±3.9
32 U	361.6 fgh	±200.5	9.1 fgh	±5.1
64 U	443.8 f	±46.9	11.2 f	±1.2
128 U	558.0 e	±39.6	14.1 e	±1.0
256 U	657.8 d	±65.0	16.6 d	±1.6
512 U	936.8 c	±55.9	23.6 c	±1.4
1024 U	1190.6 b	±98.1	30.0 b	±2.5
2048 U	1313.8 a	±42.1	33.1 a	±1.1
4096 U	1114.8 b	±30.0	28.1 b	±0.8
SEM[e]	32.25		0.81	

Main Effect of Xylanase

	linear effect	quadratic effect
reducing sugars released (μg)	$P < 0.001$	$P < 0.001$
% of total reducing sugars	$P < 0.001$	$P < 0.001$

[a] Enzymic hydrolyses were carried out in 50 mM NaAc buffer, pH 4.8, in a thermostatically controlled shaking water bath at 37 °C for 24 h. [b] Means with the same letter in the same column are not significantly different. [c] Standard deviation. [d] 100% reducing sugar was defined as the content of total acid-extractable reducing sugars in oat hulls. [e] Standard error of means.

where CEL is cellulase, $R^2 = 0.48$, RSD = 409.91, and $P < 0.001$.

$$\text{percentage of total reducing sugars released (\%)} = 15.72 + 0.04[\text{CEL}] - 0.000012[\text{CEL}]^2$$

where CEL is cellulase, $R^2 = 0.48$, RSD = 10.32, and $P < 0.001$.

Release of Reducing Sugars from Oat Hulls by *Trichoderma* Xylanase. The release of reducing sugars from oat hulls generated solely by the action of *Trichoderma* xylanase at levels ranging from 7.8 mU to 4096 U/assay was examined after a 24-h incubation, as shown in **Table 3**. The release of reducing sugars was similar and low (4−9%) with *Trichoderma* xylanase levels ranging from 7.8 mU to 32 U/assay. The percentage of total reducing sugars released was increased from 11 to 33% with increasing xylanase levels from 32 to 2048 U/assay. Further increasing *Trichoderma* xylanase levels from 2048 to 4096 U/assay resulted in no further release of reducing sugars.

Polynomial regression analysis showed a quadratic effect ($P < 0.001$) of *Trichoderma* xylanase concentration on the release of reducing sugars. The equations for estimating the release of reducing sugars are as follows:

$$\text{release of reducing sugars } (\mu\text{g}) = 299.43 + 0.99[\text{XYL}] - 0.00019[\text{XYL}]^2$$

Table 4. Release of Reducing Sugars from Oat Hulls by Cellulase, as Influenced by *Aspergillus* Ferulic Acid Esterase (FAE) and *Trichoderma* Xylanase after a 24-h Incubation[a]

cellulase level (U/assay)	xylanase level (U/assay)	FAE (mU/assay)	reducing sugars released (μg)[b]	SD[c]	% of total reducing sugars[b,d]	SD[c]
0.0625	1	13	317.6 g	±34.9	8.0 g	±0.9
2	1	13	887.7 f	±175.7	22.2 f	±4.4
16	1	13	1631.1 e	±102.7	40.1 e	±2.6
128	1	13	2204.0 d	±144.8	55.5 d	±3.7
1024	1	13	2590.0 bc	±193.7	65.3 bc	±4.9
2772.7	1	13	2373.1 cd	±49.2	59.8 cd	±1.2
0.0625	256	13	2718.2 ab	±160.5	68.5 ab	±4.0
2	256	13	2690.9 ab	±161.8	67.8 ab	±4.1
16	256	13	2445.4 bcd	±150.5	61.6 bcd	±3.8
128	256	13	2705.9 ab	±155.6	68.2 ab	±3.9
1024	256	13	2913.2 a	±161.4	73.4 a	±4.1
2772.7	256	13	1689.8 e	±198.9	42.6 e	±5.0
SEM[e]			74.62		1.88	

[a] Enzymic hydrolyses were carried out in 50 mM NaAc buffer, pH 4.8, in a thermostatically controlled shaking water bath at 37 °C for 24 h. [b] Means with the same letter in the same column are not significantly different. [c] Standard deviation. [d] 100% reducing sugar was defined as the content of total acid-extractable reducing sugars in oat hulls. [e] Standard error of means.

where XYL is xylanase, $R^2 = 0.91$, RSD = 107.14, and $P < 0.001$.

$$\text{percentage of total reducing sugars (\%)} = 7.54 + 0.02[\text{XYL}] - 0.000005[\text{XYL}]^2$$

where XYL is xylanase, $R^2 = 0.91$, RSD = 2.70, and $P < 0.001$.

Release of Reducing Sugars from Oat Hulls by Cellulase, as Influenced by *Aspergillus* Ferulic Acid Esterase and *Trichoderma* Xylanase. The release of reducing sugars from oat hulls by cellulase (at levels of 62.5 mU, 2 U, 16 U, 128 U, 1024 U, and 2772.7 U/assay), as influenced by *Aspergillus* ferulic acid esterase (at 13 mU/assay) and *Trichoderma* xylanase (at 1 U and 256 U/assay), is presented in **Table 4**. The enzyme levels for *Aspergillus* ferulic acid esterase at 13 mU/assay and *Trichoderma* xylanase at 1 and 256 U/assay were chosen on the basis of previous studies (*4*, *20*). In the presence of a low level of *Trichoderma* xylanase (1 U/assay) and *Aspergillus* ferulic acid esterase at 13 mU/assay, the release of reducing sugars was increased from 8 to 65% with increasing cellulase levels from 62.5 mU to 1024 U/assay. Further increasing the cellulase level from 1024 to 2772.7 U/assay resulted in no further increase in the release of reducing sugars. The highest release of reducing sugars (65.3%) was found at a cellulase concentration of 1024 U/assay.

In the presence of a high level of *Trichoderma* xylanase (256 U/assay) and *Aspergillus* ferulic acid esterase at 13 mU/assay, the release of reducing sugars was further increased at each level of cellulase, reaching a maximum (73.4%) with cellulase at a concentration of 1024 U/assay. Further increasing the cellulase level from 1024 to 2772.7 U/assay resulted in no further increase in the release of reducing sugars.

Polynomial regression analysis showed that, in the presence of *Aspergillus* ferulic acid esterase at 13 mU/assay, the effects of *Trichoderma* xylanase, cellulase, and *Trichoderma* xylanase × cellulase interactions and the quadratic effect of cellulase were strongly significant ($P < 0.001$) in influencing the release of reducing sugars from oat hulls. The equations for estimating

release of reducing sugars are as follow:

$$\text{release of reducing sugars } (\mu g) = 1187.44 + 5.41[XYL] + 1.84[CEL] - 0.0031[XYL \times CEL] - 0.0005[CEL]^2$$

Where CEL is cellulase, XYL is xylanase, $R^2 = 0.73$, RSD = 430.54, and $P < 0.001$.

$$\text{percentage of total reducing sugars } (\%) = 29.92 + 0.14[XYL] + 0.05[CEL] - 0.000078[XYL \times CEL] - 0.000013[CEL]^2$$

where CEL is cellulase, XYL is xylanase, $R^2 = 0.73$, RSD = 10.84, and $P < 0.001$.

DISCUSSION

The results showed that *Aspergillus* ferulic acid esterase alone had no beneficial effect on the release of reducing sugars from oat hulls. This result is in agreement with the results of Faulds and Williamson (*7*), who reported that ferulic acid esterase(III) alone from *Aspergillus niger* was unable to influence the release of reducing sugars from destarched wheat bran. This result is also consistent with previous research in our laboratory, which showed that *Aspergillus* ferulic acid esterase alone had only a minimal effect on the release ferulic acid from oat hulls. Yu et al. (*20*) showed that *Aspergillus* ferulic acid esterase alone at 13 mU/assay could release only 0.8% of total alkali-extractable ferulic acid from oat hulls. However, these authors observed that, in the presence of *Trichoderma* xylanase at 1 U and 256 U/assay, 3.8 and 15.0% of total alkali-extractable ferulic acid was released from oat hulls by *Aspergillus* ferulic acid esterase at 13 mU/assay. These results indicated a synergistic interaction between *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase on the release of ferulic acid from oat hulls.

The current research showed that, in the presence of *Aspergillus* ferulic acid esterase (13 mU/assay) and *Trichoderma* xylanase (1 U or 256 U/assay), cellulase action resulted in a significant release of reducing sugars (up to 73.4%) from oat hulls after a 24-h incubation, compared with the release of reducing sugars solely by the action of cellulase at the same level. For example, cellulase alone at 1024 U/assay released only 38.6% reducing sugars. However, cellulase at 1024 U/assay, in combination with *Aspergillus* ferulic acid esterase (13 mU/assay) and *Trichoderma* xylanase (256 U/assay), resulted in the release of 73.4% of total reducing sugars. This indicates that these enzymes act together to release reducing sugars from oat hulls. It is common to find such synergism between plant cell wall-degrading enzymes (*30*). Our results indicated that the release of reducing sugars from oat hulls by cellulase was significantly influenced by the presence of *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase.

The effect of cellulase on the efficient release of reducing sugars by adding the *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase may be attributed to the preference of *Aspergillus* ferulic acid esterase for the shorter fragments generated by the *Trichoderma* xylanase (*3*, *4*, *20*), whereas the action of *Aspergillus* ferulic acid esterase in releasing ferulic acid results in an opening of the complex cell wall structure and facilitates the accessibility of the main chain of cell wall polymers to cellulase, extending cell wall hydrolysis. Theoretically, a high release of ferulic acid and a high release of reducing sugars by the combined action of *Aspergillus* ferulic acid esterase, *Trichoderma* xylanase, and cellulase would be expected to result in a higher biodegradation of oat hulls. This will be investigated in future research.

CONCLUSION

It was concluded that, in the presence of *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase, cellulase dramatically increased the release of reducing sugars from oat hulls with a particle size of 250 μm. These results suggest that the synergistic interaction between *Aspergillus* ferulic acid esterase and *Trichoderma* xylanase on the release of ferulic acid from feruloyl polysaccharides makes the remainder of the polysaccharides open for further hydrolytic attack and facilitates the accessibility of the main chain of polysaccharides to cellulase. This action extends cell wall hydrolysis, thus releasing a higher yield of reducing sugars. Such enzymic pretreatment may provide a unique advantage to rumen microorganisms for the biodegradation of ferulic acid-containing and ferulic acid-cross-linked complex cell walls of plant materials, such as oat hulls.

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