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J Anim Sci 2009.87:1097-1105.
doi: 10.2527/jas.2008-1262 originally published online Nov 21, 2008;

A protease additive increases fermentation of alfalfa diets by mixed ruminal microorganisms in vitro[1]

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ABSTRACT: In vitro experiments were conducted to examine the characteristics and mode of action of a protease that increased the ruminal fiber digestibility of alfalfa hay. A commercial source of protease (Protex 6L, Genencor Int., Rochester, NY), already characterized for its main activities, was further analyzed to determine protease activity in response to pH, molecular size by SDS-PAGE, specificity to degrade model or feed substrates, response to autoclaving, and action of specific protease inhibitors in the absence or presence of ruminal fluid. In addition, batch culture in vitro incubations in buffered ruminal fluid were conducted to compare the enzyme product with purified protease sources, and dose-response studies (0 to 10 μL/g of forage DM) were carried out using alfalfa hay as a substrate. The enzyme product was shown to be an alkaline protease (optimum pH >8.5) of approximately 30 kDa. Specificity in the absence of ruminal fluid showed that the enzyme was active against gelatin and casein to the same extent, whereas it had limited (21% of the total) activity on BSA. In the presence of ruminal fluid and with the use of feed substrates, the protease increased ($P < 0.05$) 22-h IVDMD (%) of alfalfa hay, fresh corn silage, dry-rolled corn, and a total mixed ration composed of the 3 ingredients (39.5 vs. 44.7; 50.3 vs. 54.5; 63.8 vs. 68.4; and 55.4 vs. 56.4 for control vs. protease for each feed, respectively). Inhibitor studies in the absence of ruminal fluid indicated that the enzyme was inhibited most by a serine protease inhibitor but not by cysteine- or metalloprotease inhibitors (10 vs. 1.9 and 0.1%, respectively). In the presence of ruminal fluid, the serine protease inhibitor reversed ($P < 0.05$) the increase in alfalfa IVDMD achieved by the enzyme product, such that IVDMD was similar to that of the control treatment. Comparisons among different proteases revealed that only pure subtilisin achieved increases in IVDMD that were similar to those with protease, suggesting the serine protease was subtilisin-like (EC 3.4.1.62). Dose-response studies using alfalfa hay as substrate showed quadratic responses in IVDMD, NDF digestion, and hemicellulose and protein disappearance. It is postulated that this enzyme acts by removing structural proteins in the cell wall, allowing ruminal microbes to gain faster access to digestible substrates.

Key words: exogenous protease, fiber, in vitro disappearance, rumen digestibility

J. Anim. Sci. 2009. 87:1097–1105
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INTRODUCTION

The use of enzymes as additives for ruminant diets has traditionally been restricted to cellulases and hemicellulases (Beauchemin et al., 2003). The use of supplemental proteases in ruminant feeds has been ignored mainly because of the assumption that they would cause excessive protein degradation in the rumen, leading to inefficient N use. However, recent evidence from our group has suggested a role for proteases in improving fiber digestion in alfalfa-corn diets in vitro (Colombatto et al., 2003a,b) and in vivo (Eun and Beauchemin, 2005). In those studies, a specific protease product was selected after several screening steps, and its efficacy was tested in continuous culture (Colombatto et al., 2003b). Because of the diversity in proteases found in nature, the specific properties of the enzymes involved in the positive responses need to be elucidated to improve the formulation of enzyme additives. Type of protease involved, substrate specificity, and response of activity to pH are some of the most important factors to be considered. Further tests in the presence of ruminal fluid may help explain the mode of action of the enzyme product (**EP**) in the animal.

[1]Lethbridge Research Centre contribution No. 38708029. The authors thank A. F. Furtado, D. Vedres, and G. Hervás for excellent technical assistance, and T. Entz for help with statistical analysis.

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Received June 25, 2008.
Accepted November 4, 2008.

To this end, a series of biochemical and in vitro experiments was performed with the objective of further characterizing the protease used in previous studies. The aim was to define the type of protease present, and to describe the dose-response relationship of protease addition to alfalfa hay. Our hypothesis was that the protease would be of a type not commonly found in the rumen, and that by comparison against commercial purified proteases, the type of protease required to improve fiber digestion in the rumen would be defined.

MATERIALS AND METHODS

Biochemical Properties

The EP was a commercially available protease (Protex 6L), manufactured and supplied by Genencor Int. (Rochester, NY). The same batch was used throughout the study, within 1 yr of manufacturing. Protease activity as a function of pH was determined by using 0.4% (wt/vol) azocasein as substrate, at 39°C and with a 15-min incubation time (Colombatto et al., 2003a). Protease activity was determined at pH 5.0, 5.6, 6.2, 6.8, 7.5, and 8.0. For the pH range of 5.0 to 6.8, the buffer used was 0.1 *M* citrate-phosphate, whereas 0.1 *M* phosphate buffer was used for pH 7.5 and 8.0. The EP was diluted 100-fold before assay and 25 μL of this dilution was used. One unit of activity was defined as the absorbance given by 10 μg of a standard protease from *Streptomyces griseus* (Type XIV, P-5147; Sigma, St. Louis, MO) assayed under identical conditions. Results are expressed in equivalents per milliliter of undiluted EP.

Protein molecular mass of the EP was resolved by SDS-PAGE. The SDS-PAGE was performed following the discontinuous system of Laemmli (1970), using a Mini-Protean II kit (Bio-Rad Laboratories Ltd., Hercules, CA) and a 10% acrylamide gel. Samples were denatured in 2.5% SDS in the presence of 5% 2-mercaptoethanol by boiling for 3 min. Proteins were stained with Coomassie Blue R-250 (Bio-Rad Laboratories). Molecular weight reference markers were also obtained from Bio-Rad Laboratories.

The types of proteases present in the EP were identified by using specific protease inhibitors in a radial gel diffusion assay. Gels containing 0.5% (wt/vol) gelatin (Fisher Scientific, Fair Lawn, NJ) were prepared as described by Colombatto et al. (2003a), except that the citrate-phosphate buffer pH was 6.8. For inhibition of serine proteases, a solution (3 m*M* final concentration) of phenyl methyl sulfonyl fluoride (**PMSF**) was dissolved in 95% ethanol and added (120 μL in 10 mL total) to the liquid gel. For cysteine protease inhibition, 0.25 mL of a 0.2 m*M* solution of dichloromercuricbenzoic acid, dissolved in distilled water, was added, whereas 0.25 mL of a 1 m*M* solution of EDTA was used for inhibiting metalloproteases. Upon agar solidification, a well was made in the plate center and 5 μL of undiluted EP plus 20 μL of distilled water was added. Control treatments containing no inhibitor and ethanol only were also included to assess the effects of the inhibitors and the effects of the ethanol used to dissolve the PMSF, respectively. The plates were prepared in triplicate and incubated at 39°C for 16 h. At the end of the incubation period, the unhydrolyzed gelatin was precipitated by adding a saturated ammonium sulfate solution. The clear radial areas around the wells (denoting areas degraded by the enzymes) were measured by 2 independent observers with an electronic digital caliper (Traceable, Model No. 62379-531, Control Company, Friendswood, TX). The protease activity was then expressed in terms of millimeters of gelatin degraded, after correction for the diameter of the well. Percentage of inhibition caused by each inhibitor was determined by comparing the degraded areas in the control and in the inhibitor-containing gels.

Substrate specificity was determined by preparing gels containing 0.5% (wt/vol) gelatin, 0.5% BSA, 0.5% sodium casein, and 0.2% azocasein. All substrates were obtained from Sigma, and gel-containing plates were prepared in duplicate. Five microliters of EP, diluted in 20 μL of distilled water, was added into the plates and incubated as described above. Comparisons were made against gelatin, which was used as the standard substrate.

In Vitro Ruminal Disappearance

A series of in vitro experiments using buffered ruminal fluid were carried out to determine the substrate specificity (i.e., alfalfa hay, corn silage, or corn grain), the dose response in alfalfa diets, the influence of autoclaving the EP on the responses, and the effects of inhibitors on the EP. Finally, a comparison of EP with other purified commercial protease products was made to help determine the type of protease contributing to the positive responses in digestion.

Substrate specificity was determined by using alfalfa hay (dried and milled to pass a 4.5-mm screen), fresh corn silage (ground for 10 s with a Knifetec 1095 sample mill, Foss Tecator, Höganäs, Sweden), corn grain, and a total mixed ration (**TMR**) prepared from these components and consisting of 30% alfalfa, 30% corn silage, and 40% corn grain (DM basis). The quality and processing characteristics of these feeds are described in Colombatto et al. (2003b). Briefly, DM, CP, NDF, and ADF contents were (g/kg) 904, 417, 883, and 644; 233, 113, 101, and 143; 433, 369, 131, and 322; and 284, 178, 23.9, and 167 for alfalfa hay, corn silage, rolled corn, and the TMR, respectively. One gram of feed DM was weighed into 100-mL-capacity Duran bottles (VWR Intl., Edmonton, Alberta, Canada). Treatments for each feed consisted of no EP (control) or EP added at 1.5 μL/g of feed DM, dissolved in 1 mL of distilled water, approximately 4 h before addition of ruminal fluid. The control treatments received only 1 mL of distilled water. Forty milliliters of anaerobic buffer (Goering and Van Soest, 1970) was added to each bottle and

then placed in an incubator at 39°C for at least 2 h before ruminal fluid addition (10 mL). Ruminal fluid was obtained from 3 lactating dairy cows fed a TMR similar to that being incubated. Treatments were incubated in quadruplicate and removed after 22 h, filtered through Gooch (sintered glass) crucibles (porosity 1), and dried at 55°C for 48 h. Dry matter and OM disappearance were determined by difference. The experiment was replicated on 3 separate occasions.

The dose response to EP addition was tested by using alfalfa hay (ground through a 4.5-mm screen) as substrate. The alfalfa hay and in vitro procedures were as described above, and EP was added at 0, 0.25, 0.50, 0.75, 1, 1.5, 2.5, 4, 6, 10, and 50 μL/g of DM. Bottles were incubated in quadruplicate and removed from incubation after 22 h. Dry matter disappearance was determined as described, whereas NDF and ADF disappearance (**NDFD** and **ADFD**, respectively) were determined by using the Ankom^{200} (Ankom Corp., Fairport, NY) fiber analyzer, including α-amylase but not sodium sulfite in the NDF analysis. Hemicellulose disappearance was estimated from the difference between NDFD and ADFD. Crude protein was determined as described in AOAC (1995) method 968.06 with an autoanalyzer (Carlo Erba Instruments, Milan, Italy). The experiment was replicated on 3 separate occasions.

The influence of autoclaving the EP on the DM disappearance of alfalfa hay was determined by using native or autoclaved EP, added at 1.5 μL/g of feed DM, dissolved in 1 mL of distilled water, approximately 4 h before addition of the ruminal fluid. To determine 0-h disappearance, duplicate bottles of the control, native, and autoclaved treatments were removed and filtered immediately before ruminal fluid addition. Dry matter and CP disappearance at 0 and 22 h postincubation were determined as described above. The experiment was replicated on 2 separate occasions.

To determine whether other types of proteases could mimic the effects observed with EP, commercial sources of purified proteases (Sigma) were obtained. The EP was tested alongside trypsin (T-4799), pancreatin (P-1750), and subtilisin (P-5380), using alfalfa hay and fresh corn silage as substrates. Before the in vitro study, the protease activity of the different sources, including the EP, was determined to calculate the dose needed to supply the same amount of activity. Based on the results of the protease assay, application rates (mg/g of DM) were 1.50, 2.47, 2.80, and 0.10 for EP, trypsin, pancreatin, and subtilisin, respectively, and EP were dissolved in 1.5 mL of distilled water before addition. Control bottles containing substrate only (no enzyme) were also included. Bottles were incubated in quadruplicate and procedures were as described. Dry matter disappearance was determined for all treatments after 22 h of incubation, whereas CP disappearance (at 22 h) was determined for the control, EP, trypsin, and subtilisin treatments as described.

The effects of specific inhibitors of serine proteases on the disappearance of alfalfa hay were determined by using 3 m*M* PMSF, dissolved in 95% ethanol, and alfalfa hay as substrate. The EP was added at 1.5 μL/g of DM as described previously. The treatments, prepared in quadruplicate, were ruminal fluid and substrate only (control), ruminal fluid plus ethanol only (positive control), ruminal fluid plus inhibitor, ruminal fluid plus EP, and ruminal fluid plus EP plus inhibitor. Bottles containing ruminal fluid only (no substrate) were also included as negative controls. Bottles were incubated for 22 h at 39°C, removed, and filtered as described. Dry matter disappearance was determined, and the experiment was replicated on 2 separate occasions.

Scanning Electron Microscopy

Control and enzyme-treated alfalfa hay samples after 0 and 18 h of incubation were used to obtain scanning electron microscopy images. Samples were fixed in 4% (vol/vol) glutaraldehyde in 0.2 *M* sodium phosphate buffer (pH 7; 24 h at 4°C), and then warmed at room temperature for 1 h, followed by 3 washes in the same buffer (10 min each). Samples were then dehydrated in a graded series of ethyl alcohol (35, 50, 70, 85, and 95%) for 10 min each, followed by further dehydration in 100% alcohol (3 changes of 10 min each). Samples were then dried to a critical point with a Polaron E3100 Jumbo critical point drier (Quorum Technologies, Ringmer, East Sussex, UK) and then mounted on aluminum stubs with colloidal silver paste. Once the paste was dried, the samples were sputter-coated with a Denton Desk IV sputter coater (Denton Vacuum, Moorestown, NJ) and viewed with a Hitachi S570 scanning electron microscope (Hitachi High Technologies, Tokyo, Japan).

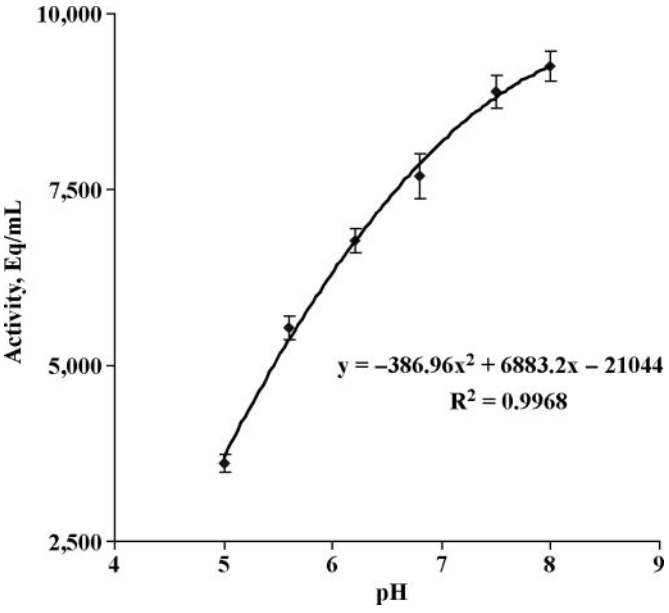

Figure 1. Protease activity of the exogenous enzyme product (Protex 6L, Genencor Int., Rochester, NY) as a function of pH. Values are expressed as equivalents of a standard (protease from *Streptomyces griseus*) per milliliter of undiluted enzyme product. Values are the average of 3 determinations, and vertical bars represent SD.

Table 1. Effect of serine-, cysteine-, and metalloprotease inhibitors on degradation of gelatin by an exogenous protease product[1]

Treatment[2]	Type of protease inhibited	Degradation, mm	SD[3]	Inhibition, % of control
Control		30.3	0.16	
Control plus ethanol		29.8	0.24	
PMSF	Serine proteases	26.5	0.61	10.8[4]
CMB	Cysteine proteases	29.7	0.10	1.9[5]
EDTA	Metalloproteases	30.2	0.48	0.1[5]

[1]Protex 6L (Genencor Int., Rochester, NY).
[2]PMSF = 3 m*M* phenyl methyl sulfonyl fluoride (dissolved in 95% ethanol with 120 μL added); CMB = 0.2 m*M* dichloromercuric-benzoic acid.
[3]n = 3.
[4]Expressed as relative to the control plus ethanol treatment.
[5]Expressed as relative to the control treatment.

At least 20 images were prepared, and the chosen images were digitally captured by using Quartz PCI software (Quartz Imaging Corporation, Vancouver, British Columbia, Canada).

Statistical Analysis

The in vitro ruminal disappearance experiments were analyzed by using PROC MIXED (SAS Inst. Inc., Cary, NC). In all cases, experimental run (i.e., replication in time) was treated as a random effect. For enzyme-substrate specificity, substrate, enzyme, and their interaction were considered as fixed effects. The effects of autoclaving were analyzed by time using autoclaving as a fixed effect. The dose response for each forage component was analyzed by linear, quadratic, and cubic contrasts. The comparison between purified proteases and the EP was analyzed using type of protease as a fixed effect. The effects of serine protease inhibitors were analyzed using enzyme as a fixed effect. In all cases, differences among means were declared as significant at $P < 0.05$, whereas trends were discussed at $P < 0.10$, unless stated otherwise.

RESULTS

Biochemical Properties

The protease activity present in the EP showed a quadratic response to pH (Figure 1), with activity being greater as pH increased. The SDS-PAGE revealed the presence of a distinct protein band of approximately 30 kDa in size (Figure 2), which is considered by the manufacturer to be responsible for 99% of the protease activity (Genencor Int., personal communication). Two other bands, smaller in comparison, were observed (approximately 15 and 22 kDa). The types of proteases present were studied by using specific inhibitors, and the results are presented in Table 1. Serine proteases were inhibited to a larger extent than were cysteine- and metalloproteases. Enzyme-substrate specificity was examined by using several substrates. The EP degraded gelatin, sodium casein, and azocasein to the same extent, whereas its action on BSA was limited (Table 2).

In Vitro Ruminal Disappearance

Overall, addition of EP increased the DM disappearance of alfalfa hay (+13%), corn silage (+8%), rolled corn grain (+7%), and the TMR (+2%; Table 3). The dose-response studies revealed quadratic ($P < 0.01$) responses in IVDMD and NDF, hemicellulose, and CP disappearances to incremental concentrations of EP (Figure 3). The greatest R^2 was detected with CP disappearance.

Autoclaving the EP destroyed its ability to influence the in vitro disappearance of alfalfa (Table 4). In contrast, native EP addition increased ($P < 0.05$) IVDMD and CP disappearance at both 0 h (i.e., preingestive ef-

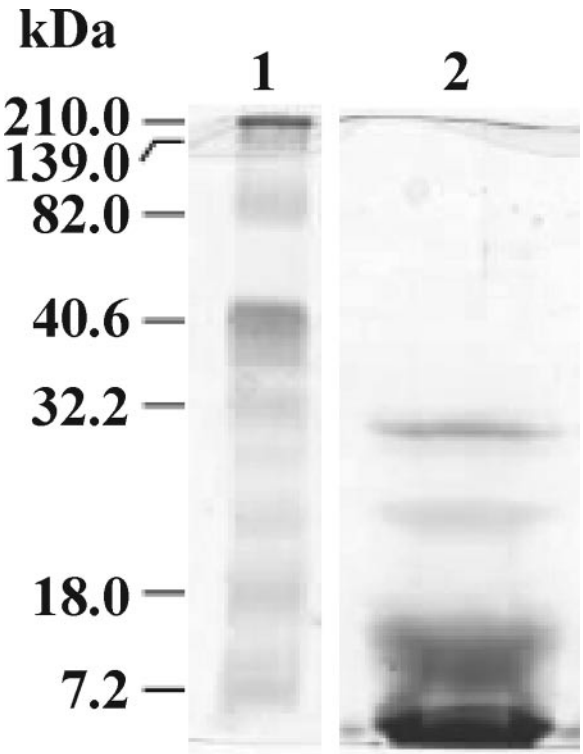

Figure 2. Sodium dodecyl sulfate-PAGE of an exogenous protease product (Protex 6L, Genencor Int., Rochester, NY). Lane 1 is a standard marker from Bio-Rad (Kaleidoscope Prestained Standards, Bio-Rad Laboratories, Hercules, CA), whereas lane 2 is the experimental sample. kDa = protein subunit size.

Table 2. Substrate specificity of an exogenous protease product[1] added at 5 μL into agar-substrate gels and incubated at 39°C for 16 h[2]

Substrate	Degradation, mm	SD[3]	Activity, % relative to activity for gelatin
Gelatin	27.6	0.44	100
Azocasein	27.1	0.60	98.1
Sodium casein	27.0	0.08	98.0
BSA	5.9	0.36	21.4

[1]Protex 6L (Genencor Int., Rochester, NY).
[2]The reaction was terminated by addition of a saturated ammonium sulfate solution.
[3]n = 2.

fects because the enzyme was added 4 h before addition of the ruminal fluid) and at 22 h postincubation (i.e., postingestive effects). An increase ($P < 0.05$) in CP disappearance was also noted at 0 h with the autoclaved treatment, but no differences ($P = 0.76$) between the control and autoclaved treatments were detected at 22 h (Table 4).

The scanning electron microscopy images are shown in Figure 4. At 0 h, some disruption of the enzyme-treated alfalfa appeared to have been initiated (Figure 4b) compared with the control samples (Figure 4a). At 18 h of incubation, disappearance was more prominent for the enzyme-treated forage (Figure 4d) than for the control forage (Figure 4c).

Of the 3 purified commercial proteases used to compare against the EP, only subtilisin increased the disappearance of alfalfa hay DM and CP at 22 h to the same extent as did EP (Table 5), although in this particular run, differences for DM disappearance did not reach significance for either alfalfa hay or corn silage. Results of the use of specific inhibitors of serine proteases are shown in Table 6. Enzyme addition increased ($P < 0.05$) IVDMD, but the inclusion of the inhibitor reversed the enzyme action, making it comparable with the control. However, adding ethanol only or the inhibitor without the EP decreased ($P < 0.05$) IVDMD with respect to the control (Table 6).

DISCUSSION

Recent research by our group has shown that an EP consisting almost exclusively of protease activity increased the in vitro ruminal fiber disappearance of diets containing alfalfa (Colombatto et al., 2003a,b). Given this somewhat unexpected response, it was important to accurately determine the type of enzyme responsible for those effects.

Results from the present study indicate that this enzyme is an alkaline protease of approximately 30 kDa in size, which has similar action against gelatin and sodium casein, but limited activity on BSA. These findings tend to agree with previous reports (Wallace and Kopecny, 1983) of large differences between the hydrolysis of casein and albumin by ruminal bacteria, suggesting that proteins with a more ordered structure, such as albumins, are relatively resistant to degradation by proteases. In addition, most of the proteolytic activity in the rumen is reportedly due to the action of cysteine-type proteases (Kopecny and Wallace, 1982; McAllister et al., 1993).

The studies using protease inhibitors in the absence of ruminal fluid suggested that the enzyme contained in EP was a serine protease, but the degree of inhibition (10%) observed when using PMSF was not high enough to prove this unequivocally. This was surprising because preliminary experiments conducted in our laboratory showed inhibitions of up to 40% using the same conditions as described here. However, in a subsequent study, Eun and Beauchemin (2005) used 10 m*M* PMSF (more than 3 times the concentration used in our study) and confirmed that the EP was a serine protease.

Additional tests were carried out to confirm the type of serine protease present in the EP. Within the differ-

Table 3. In vitro DM disappearance (g/kg; 22 h of incubation) of different feeds not treated or treated with an exogenous protease product at 1.5 μL/g of DM

	Feed[1]											
	Alfalfa hay		Corn silage		Rolled corn		Total mixed ration			Effect, *P*-value		
Item	C	T	C	T	C	T	C	T	SEM[2]	Feed	Trt[3]	Feed × Trt
IVDMD	395	447	503	545	638	684	554	564	19	0.001	0.008	0.62

[1]C = untreated feed; T = feed treated with an exogenous protease product (Protex 6L, Genencor Int., Rochester, NY).
[2]n = 3.
[3]Trt = treatment.

ent types of serine proteases, this enzyme was shown to be a subtilisin-like protease (EC 3.4.1.62). The in vitro incubations in ruminal fluid showed that only pure subtilisin, and not trypsin or pancreatin, could mimic the improvements in alfalfa hay IVDMD obtained with the experimental sample. Subtilisins are widely used in the detergent industry (Maurer, 2004) and are often regarded as having relatively low substrate specificity.

With regard to specificity when tested against ruminant feeds in the presence of ruminal fluid, the enzyme increased IVDMD of alfalfa hay, fresh corn silage, and rolled corn grain, but only minor improvements occurred for a TMR composed of these 3 ingredients. Previous studies have shown increases in alfalfa DM and fiber disappearance when using exactly the same product (Colombatto et al., 2003a,b) and large increases in in vivo digestibility of DM, OM, N, NDF, ADF, and starch in dairy cow diets (Eun and Beauchemin, 2005). Furthermore, Eun et al. (2007) found that an alkaline serine protease different from that used in our study in-

Table 4. Effects of autoclaving an exogenous protease product (application rate: 1.5 μL/g of DM) on the IVDMD and CP disappearance of alfalfa hay (g/kg) at 0 and 22 h of incubation

	Treatment			
Item	Control	Autoclaved	Native[1]	SEM[2]
0 h				
DM	230[a]	231[a]	248[b]	7
CP	360[a]	386[b]	422[c]	6
22 h				
DM	435[a]	433[a]	472[b]	14
CP	449[a]	452[a]	573[b]	7

[a–c]Means within rows followed by different superscripts differ ($P < 0.05$).

[1]Native = not autoclaved exogenous protease (Protex 6L, Genencor Int., Rochester, NY).

[2]n = 8.

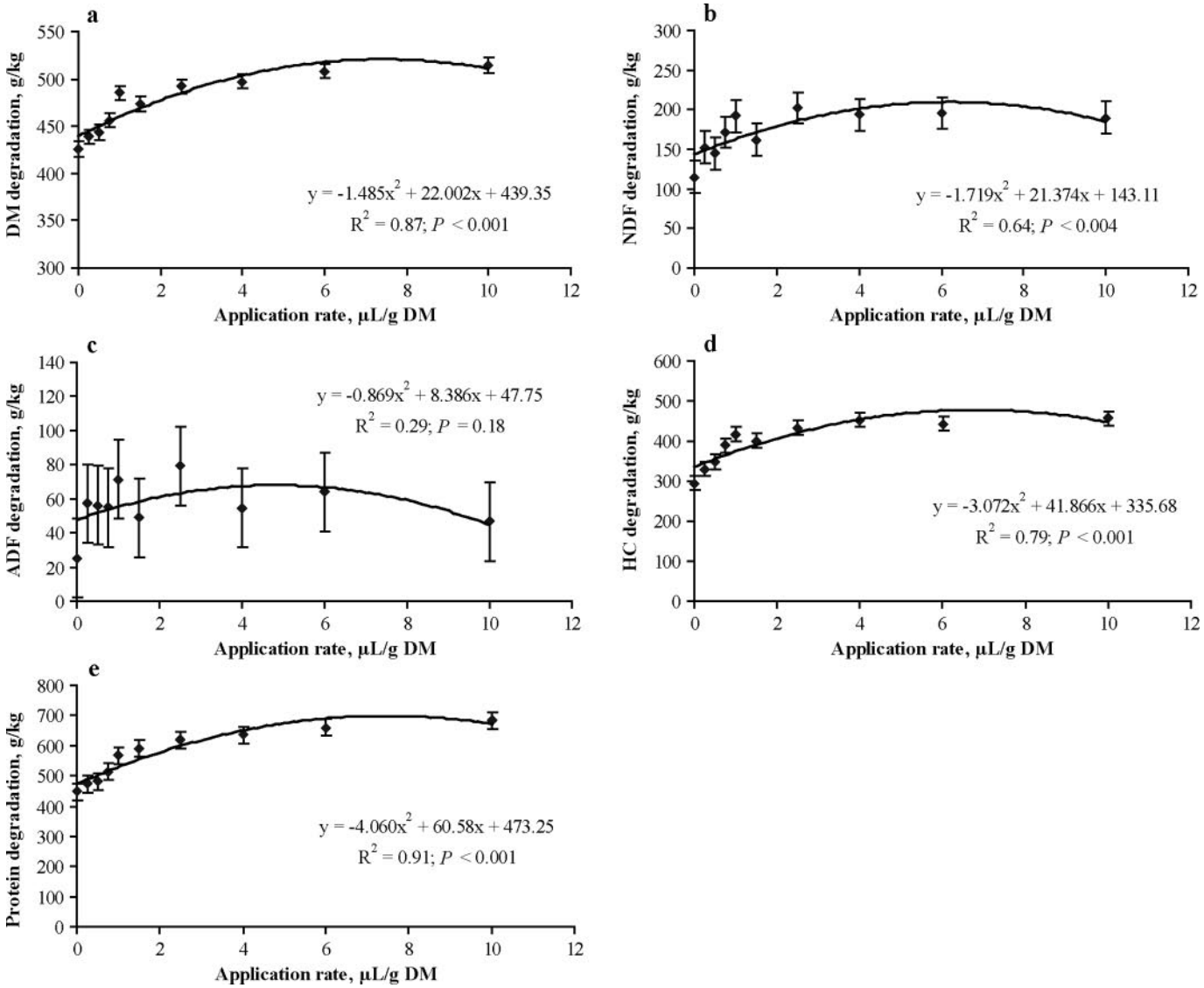

Figure 3. Dose response of addition of exogenous protease (Protex 6L, Genencor Int., Rochester, NY) to alfalfa hay on DM (a), NDF (b), ADF (c), hemicellulose (HC; d), and CP (e) degradation at 22 h postincubation with ruminal microbes. Vertical bars represent SEM.

creased the gas production, IVDMD, and NDFD of alfalfa hay, in contrast to an acidic protease, which failed to show any effect.

Another study from Eun and Beauchemin (2007) used papain (EC 3.4.22.2) and found increases in gas production, IVDMD, and NDFD of alfalfa hay and corn silage. The fact that EP increased IVDMD in corn silage was surprising, given that previous studies with EP have failed to show any effect (Colombatto et al., 2003a). However, in the present study we used fresh, as opposed to dried and ground, corn silage, which may have influenced the results. With rolled corn grain, the observed increase could be attributed to a disruption of the protein matrix that surrounds the starch granule by the EP, allowing faster microbial colonization of the substrate (McAllister et al., 1993).

Given that responses to EP addition were largest for alfalfa hay, we chose this forage for a dose-response study. A wide range of enzyme application rates (0 to 10 μL/g of forage DM) was used to best describe the nature of the relationship. The observed quadratic responses are in agreement with previous in vivo studies using alfalfa (Beauchemin et al., 1995), and may be partly explained by a progressive competition between

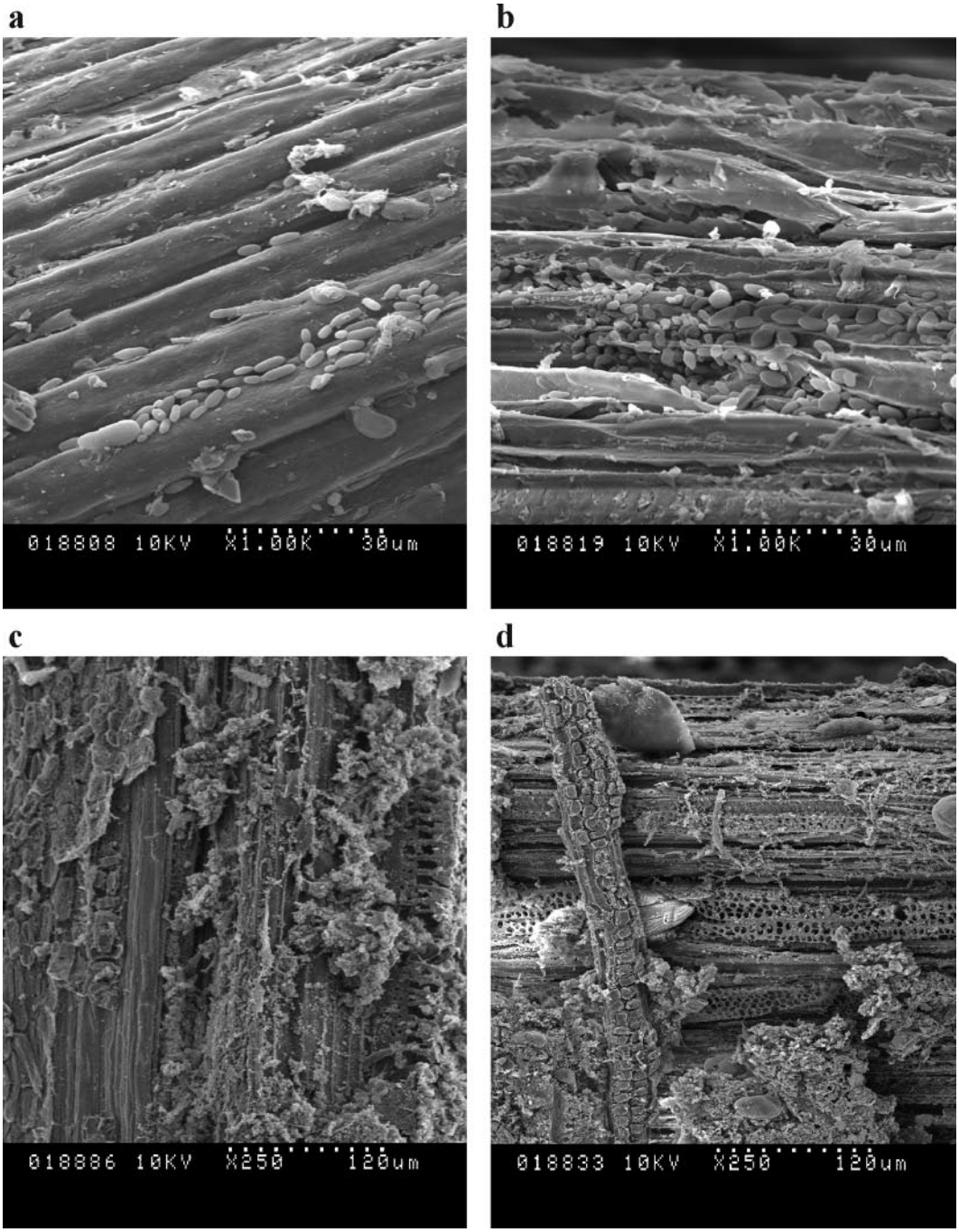

Figure 4. Scanning electron microscopy images of alfalfa hay samples, untreated (a) or enzyme-treated (b; Protex 6L, Genencor Int., Rochester, NY) at 0 h, or untreated (c) or enzyme treated (d) at 18 h postincubation with ruminal fluid in vitro.

Table 5. Comparison of an exogenous protease product[1] with purified commercial sources of trypsin, pancreatin, and subtilisin, on their effects on the IVDMD and CP disappearance of alfalfa hay (g/kg) and corn silage at 22 h of incubation

	Treatment[2]					
Substrate	Control	EP	PANC	SUBT	TRYP	SEM[3]
Alfalfa hay						
DM	409	436	396	438	411	15
CP	485[a]	592[c]	ND[4]	528[b]	460[a]	20
Corn silage						
DM	548	553	552	569	535	16
CP	137[b]	292[c]	ND	274[c]	54[a]	27

[a–c]Means within rows followed by different superscripts differ ($P < 0.05$).
[1]Protex 6L (Genencor Int., Rochester, NY).
[2]Control = no enzyme added; EP = enzyme product; PANC = pancreatin; SUBT = subtilisin; TRYP = trypsin.
[3]n = 4.
[4]ND = not determined.

endogenous and exogenous enzymes for binding sites (Morgavi et al., 2004). The increase in protein disappearance with increasing EP addition may be regarded, in some cases, as unfavorable for dairy cows. Aufrerè et al. (1994) indicated that N in alfalfa NDF represented 11% of the total N, and despite their suggestion that that quantity was too small to have a bearing on the degradability of total protein, it could be argued that the concomitant fiber digestion might have been achieved because of the removal of these cell wall proteins. In addition, a diet could be reformulated to accommodate this increase in rate of protein disappearance of alfalfa in the rumen when using the exogenous protease as an additive to increase fiber digestion.

To further confirm that the responses to EP were due to enzymatic effects, the product was autoclaved and the response was compared with that obtained with the native protease. The action of EP in the presence of ruminal fluid was inactivated by autoclaving, indicating that the stimulatory agent was heat sensitive. Sensitivity to heat is strong evidence of an enzyme effect or, less likely, a heat-labile nutrient (Wallace et al., 2001). Moreover, in subsequent enzyme assays in our laboratory, we confirmed that all enzymatic activity had been destroyed by autoclaving. The small increase in CP disappearance at 0 h with the autoclaved enzyme compared with the control is difficult to explain, but despite this difference, 0-h CP disappearance was much greater with the native enzyme than with the other treatments.

The scanning electron microscopy images illustrate the differences observed between control and enzyme-treated alfalfa samples at both 0- and 18-h incubation times. Enzyme-treated samples showed large disrupted areas that could be used by bacteria to attach and colonize the substrate rapidly, allowing the fermentation to begin earlier (Beauchemin et al., 2003). At 18 h postincubation, the large disrupted areas seemed to support the observed increases in IVDMD, NDFD, ADFD, and hemicellulose and protein disappearance noted in the accompanying studies.

The use of specific protease inhibitors in the presence of ruminal fluid showed that when a serine protease inhibitor was included, there was no effect on alfalfa hay IVDMD. This inhibitory response is an indication that the EP was likely a serine protease. However, the fact that ethanol or the inhibitor (which was dissolved in minimal amounts of ethanol) alone showed less IVDMD than the control prevents this from being conclusive. From these results, 2 possibilities exist: the enzyme was inactivated by adding ethanol or inhibitor to the sample, or the enzyme was still effective on the alfalfa,

Table 6. Effects of serine proteases inhibitors (3 m*M* phenyl methyl sulfonyl fluoride) on the IVDMD of alfalfa hay (g/kg; 22 h of incubation), with or without addition of the exogenous protease product[1] (1.5 μL/g of DM)

	Treatment[2]					
Item	Control	EP	EP+ETH$^+$INH	ETH	ETH$^+$INH	SEM[3]
IVDMD	390[b]	426[c]	388[b]	358[a]	349[a]	10

[a–c]Means within rows followed by different superscripts differ ($P < 0.05$).
[1]Protex 6L (Genencor Int., Rochester, NY).
[2]Control = untreated; EP = enzyme product; EP+ETH$^+$INH = enzyme plus ethanol plus inhibitor; ETH = ethanol; ETH$^+$INH = ethanol plus inhibitor.
[3]n = 8.

allowing the IVDMD to equal that of the control despite the negative effects of the ethanol, the inhibitor, or both. It is important to allow for the possible effects of the added ethanol when evaluating the data. More detailed work would be required to prove that the EP was a serine protease, although strong evidence for this is shown in the present study.

Colombatto et al. (2003a,b) hypothesized that the mode of action of alkaline serine proteases in ruminant diets was related to the removal of structural barriers, allowing the ruminal microorganisms to access digestible nutrients. These barriers could be composed of lignified middle lamella or primary walls, which would prevent or delay microbial access for disappearance (Jung et al., 2000).

Alternatively, as suggested by Wallace and Kopecny (1983), these barriers could be composed of structural proteins from the cell wall, which might act as barriers to microbial digestion. It has been established that alfalfa plants contain large amounts of wall protein (Bacic et al., 1988), and tyrosine residues in those wall proteins could be cross-linking lignin with polysaccharides (Jung, 1997). One of the major proteins found in primary walls of dicotyledonous plants is extensin, which consists of almost 50% hydroxyproline. Extensin is believed to be cross-linked via isodityrosine linkages (Fry, 1986), whereas more recent work has suggested the existence of pectin-protein, protein-protein, and even protein-phenolic-protein cross-links between extensins (Qi et al., 1995). If the subtilisin-like protease used in this study targets these cross-links, then microbial access could be increased and fermentation would proceed faster. It has been suggested that tyrosine residues may be the recognition site for some serine proteases such as chymotrypsin (Means and Feeney, 1971; Kopecny and Wallace, 1982), which further supports this idea.

In conclusion, increasing doses of a protease enzyme increased IVDMD, NDFD, and the disappearance of hemicelluloses and protein of alfalfa hay in a quadratic fashion, and the response was inactivated by autoclaving. The enzyme was characterized as an alkaline, serine-type protease, with a main subunit protein size of 30 kDa. The enzyme appeared to be subtilisin-like when compared with purified pure serine proteases. It is postulated that this enzyme acts by removing structural proteins in the cell wall, allowing faster access of ruminal microbes to digestible substrates.

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