J. Dairy Sci. 102:10925–10933
https://doi.org/10.3168/jds.2019-16436

The effect of various doses of an exogenous acid protease on the fermentation and nutritive value of corn silage

Michelle C. Der Bedrosian* and Limin Kung Jr.†
Department of Animal and Food Sciences, University of Delaware, Newark 19716-1304

ABSTRACT

The objective of this experiment was to evaluate the effects of treating whole-plant corn at harvest with various doses of an exogenous acidic protease on fermentation and changes in nutritive value after a short period (45 d) of ensiling. Whole-plant corn (37% dry matter) was chopped and treated with 0, 20, 200, 1,000, or 2,000 mg of protease/kg of wet forage. Forages (~500 g) were packed in bag silos and ensiled at 22 to 23°C for 45 d. Data were analyzed as a 5 × 2 factorial arrangement of treatments with the main effects of the dose of protease, day of ensiling, and their interaction. Treatment with protease did not alter the concentrations of dry matter, neutral detergent fiber, acid detergent fiber, starch, lactic acid, or acetic acid compared with untreated silage, with the exception that the concentration of starch was lower in silage treated with 20 mg of protease/kg compared with untreated silage. However, the 2 highest doses of protease resulted in silages with higher concentrations of ethanol and more yeasts compared with untreated silage. Protease treatment did not affect the ruminal in vitro digestibility of neutral detergent fiber. Concentrations of soluble protein (percentage of crude protein) increased after ensiling for all treatments but was not different between silage treated with the lowest dose of protease and untreated silage. Soluble protein increased in a dose-dependent manner above the low dose of protease in silages. Concentrations of NH_3-N were higher only in silages treated with the 2 highest doses of protease compared with untreated silage. Silages treated with the 3 highest doses of protease were higher in ruminal in vitro digestibility of starch compared with untreated silage but were similar to each other. The concentrations of total AA were determined in fresh forage and silages for the untreated and 200 and 2,000 mg/kg doses of protease. Neither amount of added protease affected the total concentrations of essential, nonessential, or total AA in silage. However, of the essential AA, treatment with protease resulted in silages with lower concentrations of lysine and arginine but higher concentrations of leucine compared with untreated silage. The 200 mg/kg dose of protease substantially improved ruminal in vitro starch digestion in corn silage after a short period of ensiling without affecting concentrations or numbers of ethanol and yeasts, respectively.
Key words: protease, starch digestibility, corn silage

INTRODUCTION

Many types of enzymes have been used as feed or silage additives to improve the nutritive value of forages for dairy cows. Most of these additives have had various carbohydrase activities that are essential in metabolizing plant cell-wall fiber (Sheperd and Kung, 1996; Kung et al., 2000) or starch (Klingerman et al., 2009; McCarthy et al., 2013). Some research has been conducted with protease enzymes as feed additives for ruminants (Eun and Beauchemin, 2005; DePeters et al., 2007), but proteases have not been commonly used as silage additives because excessive proteolysis occurs naturally during ensiling. However, in corn silage, natural proteolytic mechanisms acting on the prolamin–starch matrix have been implicated (Hoffman et al., 2011) as the cause of increased ruminal in vitro starch digestibility (**IVSD**) observed with time of ensiling (Philippeau and Michalet-Doreau, 1998; Allen et al., 2003; Der Bedrosian et al., 2012). Junges et al. (2017) reported that 60 and 30% of total proteolytic activity in the corn kernel was from bacterial and plant origin, respectively. Because IVSD is relatively low in fresh forage compared with corn silage that is stored for several months, recommendations suggest that corn silage should be ensiled for 4 to 6 mo before feeding (Ward and de Ondarza, 2008; Hallada, 2009); however, many dairies do not have adequate inventory or silos to follow this guideline and must feed silage soon after harvest. As a potential way to overcome this challenge, exogenous protease enzymes have been added to corn silage

Received February 5, 2019.
Accepted July 30, 2019.
*Current address: Vita Plus Corporation, PO Box 259126, 2514 Fish Hatchery Road, Madison, WI 53725-9126.
†Corresponding author: lksilage@udel.edu

(Young et al., 2012; Windle et al., 2014; Ferraretto et al., 2015). Young et al. (2012) reported that addition of exogenous acid proteases at harvest to whole-plant corn accelerated the increase in potentially available ruminal starch that occurs due to normal ensiling. However, in that study, only low (20 mg of protease/kg of wet forage) and high (2,000 mg of protease/kg of wet forage) concentrations of proteases were tested, and there was no evaluation of changes in the concentrations of AA due to treatment with protease. In a study by Windle et al. (2014), a high level of acid protease (2,000 ppm) stimulated the growth of yeasts in silage, which is a potentially harmful consequence because some yeasts can initiate aerobic spoilage in silages (Pahlow et al., 2003). We hypothesized that a low dose of an exogenous protease could improve ruminal IVSD after a short time of ensiling without adversely increasing yeasts that can produce large quantities of ethanol and result in silages that are less aerobically stable. Thus, the objective of this study was to evaluate several doses of an acid protease added to corn plants at harvest on silage fermentation and nutritive value.

MATERIALS AND METHODS

Corn plants (Mycogen hybrid A6867; Mycogen Seeds, Indianapolis, IN) grown at the University of Delaware (Newark) were harvested by hand from 5 random locations in a single field. Plants were chopped to a theoretical length of 19 mm using a pull-type chopper equipped with a kernel processer (John Deere 3975, Moline, IL). Five replicated piles of forage were prepared from each of the 5 locations to yield 25 total piles. Freshly chopped corn was not inoculated with any inoculant. One replicated pile from each area was treated separately with (1) a 0.1 *M* phosphate buffer (pH 5.5, 5% vol/wt of fresh forage; **CT**); (2) buffer with protease (AB Vista, Wiltshire, UK), resulting in 20 mg of an experimental protease formulation/kg of wet forage (**E1**); (3) buffer with protease, resulting in 200 mg/kg of wet forage (**E2**); (4) buffer with protease, resulting in 1,000 mg of a protease formulation/kg of wet forage (**E3**); or (5) buffer with protease, resulting in 2,000 mg of a protease formulation/kg of wet forage (**E4**). The buffer solutions were applied via separate spray bottles to the piles of forage while mixing. Buffer was used rather than water to encourage solubility. The protease used in this experiment was the same E85 formulation (AB Vista; derived from *Aspergillus niger*) used in the study by Young et al. (2012). It was an experimental food-grade acidic aspartic protease with no fibrolytic or amylolytic activity. The protease formulation had 516 U of activity/mg of solids, where 1 U of activity produced a change in absorbency at 280 nm of 0.001/min at pH 3.6 and 22°C, measured as trichloroacetic acid-soluble products using hemoglobin as a substrate. Activity of protease decreased as the pH of the assay increased above a pH of 3.6 (Figure 1). After application of protease, approximately 500 g of treated forage from each pile was packed in nylon-polyethylene pouches (3.5-mil thickness, 15.2 × 30.5 cm; Doug Care Equipment Inc., Springville, CA), vacuumed, and heat sealed with a Fast Vac vacuum machine (ABC Office, Kaysville, UT). Bags were stored in the laboratory between 22 and 23°C and ensiled for 45 d. Fresh forage from each pile served as replicate samples for d 0.

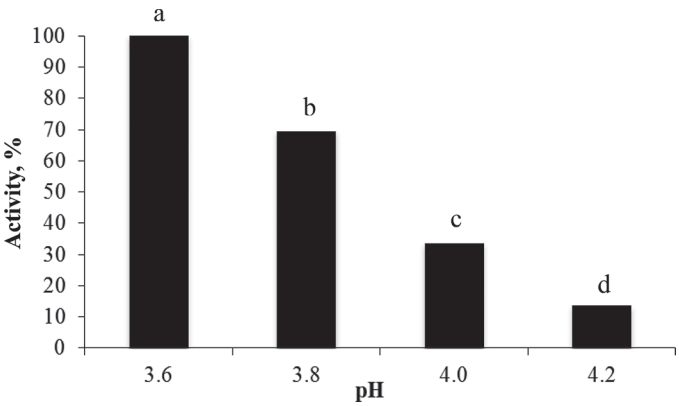

Figure 1. The effects of pH on proteolytic activity of the acidic protease, expressed as a percentage of activity at pH 3.6 at 22°C (100%) for the protease enzyme used in the study. The enzyme contained 516 U of activity/mg of solids at pH 3.6. One unit produced a change in absorbency at 280 nm of 0.001/min at the pH and temperature used, measured as trichloroacetic acid-soluble products using hemoglobin as a substrate. SEM = 3.35. Means with different letters (a–d) differ ($P < 0.05$).

Water extracts were prepared for freshly treated forages and silages by obtaining 25 g of plant material with 225 mL of sterile Ringer solution (Oxoid BR0052G; Oxoid Ltd., Cambridge, UK) and homogenizing for 1 min on a medium setting in a Proctor-Silex 57171 blender (Hamilton Beach, Washington, NC). A portion of the homogenate was filtered through Whatman 54 filter paper (Whatman Ltd., Florham, NJ) and acidified with 50% H_2SO_4 to reduce the pH of the extract to 2.0, and the water extracts were frozen (−20°C) until further analysis. Water extracts were later analyzed for NH_3-N (Okuda et al., 1965), water-soluble carbohydrates (Nelson, 1944), and lactic acid, acetic acid, and ethanol (Muck and Dickerson, 1988). A portion of each water extract was unacidified and used for enumeration of lactic acid bacteria by pour-plating on de Man, Rogosa and Sharpe agar (Oxoid CM361; Oxoid) and yeasts and molds using malt extract agar (Oxoid CM0059; Oxoid). Plates were incubated aerobically at

32°C for 48 to 72 h, and plates containing between 30 and 300 cfu were counted.

Fresh forages and silages were analyzed for DM content by drying samples in a forced-air oven at 60°C for 48 h. A portion of each dried sample was ground through a 1-mm screen using an Udy Cyclone sample mill (Udy Corp., Fort Collins, CO) and was used for the analysis of fiber residues, total N, and soluble protein (**SP**). Neutral detergent fiber was quantified using the procedures of Van Soest et al. (1991) using amylase and sodium sulfite. Acid detergent fiber was quantified in ground samples according to the procedure described by Goering and Van Soest (1970), with the modification that the fiber residue from the ADF was recovered on a 1.5-μm particle retention 7-cm Whatman glass fiber filter in a California Buchner funnel instead of a Gooch crucible to allow for better filtration. Analysis of NDF and ADF was not sequential, and quantities of NDF and ADF were presented on a DM (not ash-free) basis. Total N was determined by combustion of the sample (Leco CNS 2000 analyzer; Leco Corp., St. Joseph, MI), and CP was calculated by multiplying the resulting total N by 6.25 (method 990.03; AOAC International, 2006). A separate portion of the dried sample was ground through a 3-mm screen using an Udy Cyclone sample mill (Udy Corp.) and was used for the analysis of SP and starch. The amount of SP (as a percentage of CP) was also determined on 3-mm ground samples according to the methodology described by Krishnamoorthy et al. (1982). The starch content of each sample was determined and corrected for free glucose according to the method described by Hall (2009).

Ruminal in vitro 30-h digestibility of NDF (ground to pass a 2-mm screen; Udy Cyclone sample mill, Udy Corp.) was determined according to the methodology described by Tilley and Terry (1963). A 7-h IVSD assay was performed (Tilley and Terry, 1963) on samples ground through a Wiley mill (Thomas Scientific, Swedesboro, NJ) to pass through a 3-mm screen. All samples for each in vitro determination were run in duplicate and replicated on 3 separate days. Ruminal fluid was pooled from 3 lactating cows with rumen fistula that were fed a standard diet comprising 25% corn silage, 25% alfalfa haylage, and 50% concentrate (DM basis). Both NDF digestibility and IVSD were conducted by Cumberland Valley Analytical Labs (Waynesboro, PA).

The protease was assayed for activity at pH 3.6, 3.8, 4.0, and 4.2 at 22°C using hemoglobin as the substrate (Sigma-Aldrich, St. Louis, MO). Hemoglobin is the recommended substrate when assays are conducted under acidic conditions because casein has a pH of 4.8 and precipitates below a pH of 6 (Sumantha et al., 2006). Proteolytic activity was expressed as a percentage of activity at pH 3.6.

The AA contents of fresh forages on d 0 and silages on d 45 from treatments CT, E2, and E4 were determined by the Experimental Station Chemical Laboratories of the University of Missouri (Columbia, MO) according to AOAC International (2006) method 982.30.

The data were analyzed as a completely randomized design with a 5 × 2 factorial arrangement of treatments with factors including 5 levels of protease (CT, E1, E2, E3, and E4; fixed effect) and 2 d of ensiling (0 and 45 d; fixed effect). Data were analyzed using the Fit Model procedure of JMP version 8.02 (SAS Institute Inc., Cary, NC), and differences were reported as significant when $P \leq 0.05$. Main effects were protease dose, days of ensiling, and their interaction. Means were separated by Tukey test ($P \leq 0.05$; Snedecor and Cochran, 1980). Relationships between IVSD and soluble protein and between ammonia-N and soluble protein were determined using the Fit X by Y procedure of JMP.

For enzyme activity, data were analyzed using the Fit Model procedure of JMP version 8.02 (SAS Institute Inc.). Means were separated by Tukey test ($P \leq 0.05$; Snedecor and Cochran, 1980).

RESULTS

Table 1 shows the chemical composition of fresh forages (after treatment with protease but before ensiling) and resulting silages. The concentrations of DM were similar among all treatments in fresh forage and silage. There was an interaction between protease dose and day of ensiling for CP, but the differences among treatments were small. There was also an interaction between protease dose and day of ensiling on the concentrations for SP and NH_3-N. On d 0, SP ranged from 30.45% to 38.50% and, compared with untreated forage, was affected only by treatment with E4 (lower than all other treatments). The concentration of SP for CT increased from 35.30% in fresh forage to 44.08% in silage. Treatment with E1 resulted in a similar increase in SP with ensiling and was not different from CT. However, higher doses of protease resulted in progressive increases in the SP of silages (E2 = 59.10%, E3 = 67.72%, and E4 = 71.60%). The concentrations of NH_3-N in fresh forages were similar among all treatments, but after ensiling they were higher in E3 (0.082%) and E4 (0.096%) compared with CT (0.044%). The concentrations of NDF were unaffected by day of ensiling or dose of protease. There were minor differences in the concentrations of ADF between each day, but the dose level of the protease did not affect this measurement. Overall, the concentration of starch in fresh forages (37.51%) was greater than that in the resulting silages

Table 1. Chemical composition (% of DM, unless otherwise stated) of fresh corn plants (d 0; after treatment but before ensiling) and ensiled corn plants (d 45) that were treated with various doses of an exogenous acidic protease

Item[1]	DM	CP	SP,[2] % of CP	NH_3-N	NDF	NDF-D,[3] % of NDF	ADF	Starch
d 0								
CT	36.2	7.82^{a}	35.3^{ef}	0.050^{b}	34.6	59.88	19.5	39.3
E1	34.9	7.44^{ab}	35.0^{f}	0.054^{b}	38.6	60.08	20.6	33.9
E2	35.7	7.50^{ab}	38.5^{e}	0.034^{b}	35.1	60.56	18.8	39.3
E3	35.1	7.54^{ab}	36.9^{ef}	0.038^{b}	36.2	60.12	19.5	37.3
E4	36.5	7.26^{b}	30.5^{g}	0.036^{b}	36.0	59.00	19.3	37.7
d 45								
CT	34.4	7.66^{ab}	44.1^{d}	0.044^{b}	35.7	56.38	19.7	36.5
E1	33.8	7.70^{ab}	46.0^{d}	0.050^{b}	36.7	56.50	20.9	34.8
E2	34.63	7.42^{ab}	59.1^{c}	0.056^{b}	35.3	57.06	20.5	37.7
E3	34.7	7.26^{b}	67.7^{b}	0.082^{a}	36.4	55.68	20.5	35.6
E4	35.0	7.60^{ab}	71.6^{a}	0.096^{a}	36.4	55.80	20.8	34.4
Day means								
d 0	35.7^{a}	7.51	34.7	0.040	36.3	59.93^{a}	19.6^{b}	37.5^{a}
d 45	34.5^{b}	7.51	58.2	0.066	36.1	56.28^{b}	20.5^{a}	35.8^{b}
Treatment means								
CT	35.3	7.73	39.7	0.042	35.1	58.13	19.6	37.9
E1	34.3	7.54	39.9	0.052	38.0	58.29	20.8	34.3
E2	35.2	7.46	48.8	0.045	35.2	58.81	19.6	38.5
E3	34.9	7.40	52.3	0.060	36.3	57.90	20.0	36.5
E4	35.7	7.41	49.8	0.066	36.2	57.40	20.1	36.1
SEM	0.47	0.11	1.08	0.002	0.82	0.45	0.48	1.05
P-value[4]								
Pro	0.07	<0.01	<0.01	<0.01	0.06	0.06	0.17	<0.01
Day	<0.01	0.85	<0.01	<0.01	0.97	<0.01	<0.01	<0.01
Pro × day	0.66	0.01	<0.01	<0.01	0.52	0.71	0.43	0.36

$^{a–g}$Means within a column with different superscripts differ ($P < 0.05$).

[1]CT = control (no protease addition); E1 = treatment to yield 20 mg of a protease formulation/kg of wet forage; E2 = treatment to yield 200 mg of a protease formulation/kg of wet forage; E3 = treatment to yield 1,000 mg of a protease formulation/kg of wet forage; E4 = treatment to yield 2,000 mg of a protease formulation/kg of wet forage.

[2]Soluble protein.

[3]In vitro ruminal digestibility of NDF (24 h).

[4]Pro = effect of protease dose; day = effect of day of ensiling.

(35.83%). Specifically, in fresh forage and silage, the concentrations of starch were lower in E1 compared with CT. However, higher doses of proteases did not affect the concentrations of starch at any time point.

There was an interaction between different doses of protease and day of ensiling on IVSD (Figure 2). In fresh forage, E3 was the only dose of protease that resulted in a greater IVSD (67.19%) compared with CT (59.72%). Overall, ensiling resulted in an increase in IVSD for all treatments. However, it was greater in E2 (80.73%), E3 (83.56%), and E4 (82.98%; but similar among each other) compared with CT (74.51%). Treatment with the lowest dose of protease, E1, did not affect IVSD in silage compared with CT.

The AA profiles of CT, E2, and E4 are shown in Table 2. Overall, there were no effects of protease dose on the concentrations of total EAA, total NEAA, and total AA. Ensiling did not affect the concentrations of total NEAA or AA, but it resulted in slightly lower concentrations of EAA in silage (decreasing from 2.74% to 2.63%). Of the EAA, the concentrations (as a percentage of total AA) of histidine, isoleucine, and tryptophan increased with ensiling, and the concentrations of arginine and threonine decreased. Of the NEAA, the concentrations of alanine, hydroxy lysine, ornithine, and proline increased with ensiling, whereas the concentrations of aspartic acid, glutamic acid, glycine, hydroxyproline, serine, and tyrosine decreased. After 45 d, treatment with protease resulted in reduced concentrations of lysine and arginine but increased concentrations of leucine compared with untreated silage. Only treatment with E4 increased concentrations of phenylalanine relative to untreated silage.

The fermentation profiles and microbial compositions of fresh forages and silages are shown in Table 3. Concentrations of water-soluble carbohydrates decreased with ensiling but were similar among treatments within fresh forages and silages. The pH of fresh forages ranged from 5.61 to 5.68 and was similar among treatments. After ensiling, the pH of silages ranged from 3.66 to 3.71, and although there were some differences among treatments, no dose of protease differed from CT. The

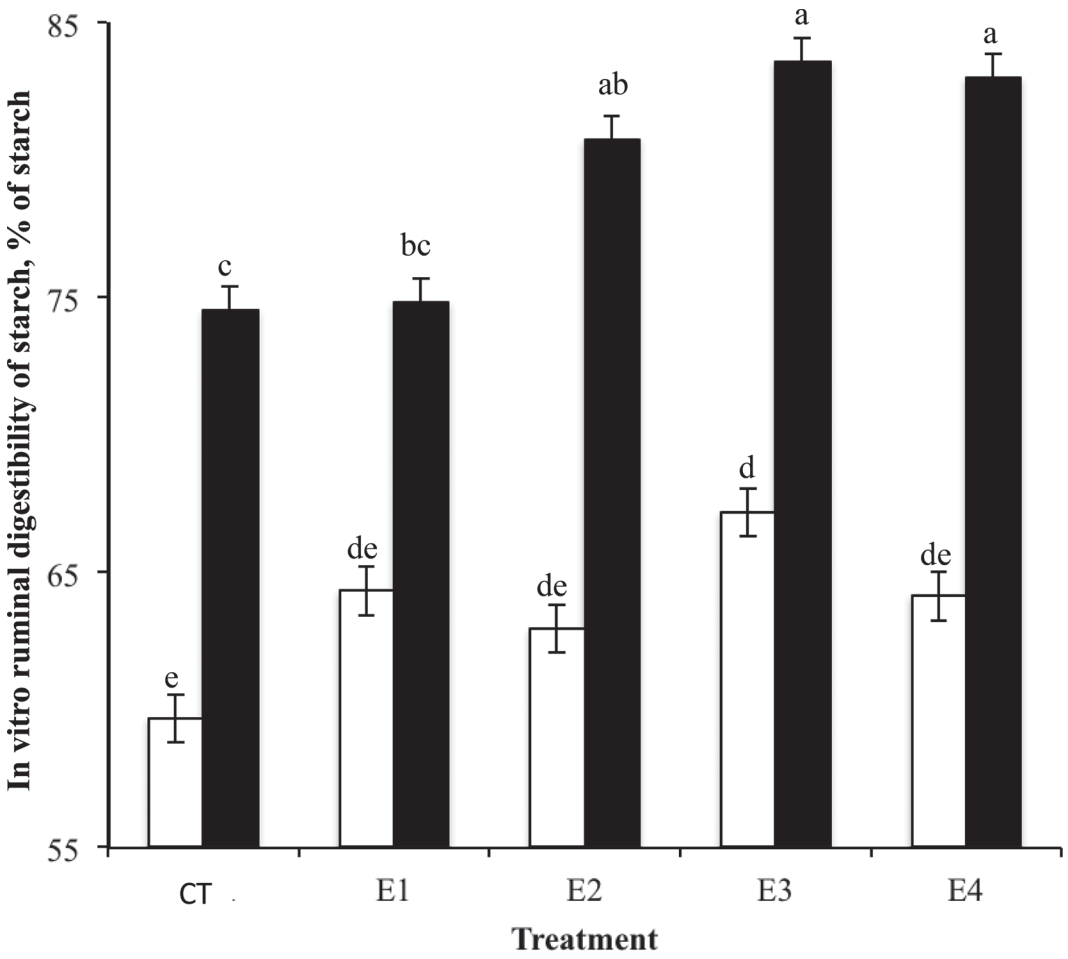

Figure 2. The 7-h digestibility of starch (% of starch) of corn silages that were treated separately with (1) a 0.1 *M* phosphate buffer (pH 5.5, 5% vol/wt of fresh forage; CT); (2) a 0.1 *M* phosphate buffer (pH 5.5, 5% vol/wt of fresh forage) with protease, resulting in 20 mg of a protease formulation/kg of wet forage (E1); (3) a 0.1 *M* phosphate buffer (pH 5.5, 5% vol/wt of fresh forage) with protease, resulting in 200 mg/kg of wet forage (E2); (4) a 0.1 *M* phosphate buffer (pH 5.5, 5% vol/wt of fresh forage) with protease, resulting in 1,000 mg of a protease formulation/kg of wet forage (E3); or (5) a 0.1 *M* phosphate buffer (pH 5.5, 5% vol/wt of fresh forage) with protease, resulting in 2,000 mg of a protease formulation/kg of wet forage (E4). The 7-h digestibility of starch after treatment but before ensiling (white bars) and after 45 d of ensiling (black bars) is shown. Means with different letters (a–e) differ ($P \leq 0.05$). SEM = 0.88. Main effect of protease dose, $P < 0.01$; main effect of day of ensiling, $P < 0.01$; interaction between protease dose and day of ensiling, $P < 0.02$. Error bars denote SEM.

concentrations of lactic and acetic acids in silage were unaffected by any dose of protease. In contrast, the concentrations of ethanol in silages were highest for E4 (1.85%), followed by E3 (0.85%), both being greater than CT (0.47%). Lower doses of protease did not affect the concentrations of ethanol in silages. In fresh forages, the numbers of lactic acid bacteria were similar among all doses of proteases. Numbers of lactic acid bacteria increased with ensiling to an equal extent among all treatments. There was an interaction between days of ensiling and treatment with proteases for numbers of yeasts. The numbers of yeasts were similar among all treatments in fresh forages, but after ensiling they were higher in E3 and E4 compared with CT.

DISCUSSION

We found that all forages in this study fermented well, resulting in a low pH with typical concentrations of fermentation acids and ethanol. As in previous studies, ensiling resulted in silages with higher IVSD compared with fresh forage (Philippeau and Michalet-Doreau, 1998; Allen et al., 2003; Der Bedrosian et al., 2012). Similar to the results reported by Young et al. (2012), treatment with proteases did not affect the concentrations of lactic or acetic acids. However, in the current study, high doses (1,000 and 2,000 mg/kg) of proteases resulted in corn silages with more yeasts and higher concentrations of ethanol compared with un-

Table 2. The AA profile (% of total AA, unless otherwise stated) of fresh corn plants (d 0; after treatment but before ensiling) and ensiled corn plants (d 45) that were treated with various doses of an exogenous acidic protease formulation[1]

	d 0			d 45			Day means		Main effect treatment means				*P*-value[2]		
Item	CT	E2	E4	CT	E2	E4	d 0	d 45	CT	E2	E4	SEM	Day	Pro	Day × pro
EAA															
Arginine	4.97^{a}	4.82^{a}	4.81^{a}	3.26^{b}	2.54^{c}	1.93^{d}	4.87	2.58	4.12	3.68	3.37	0.05	<0.01	<0.01	<0.01
Histidine	2.42	2.41	2.39	2.55	2.58	2.54	2.41^{b}	2.56^{a}	2.49	2.49	2.47	0.03	<0.01	0.66	0.83
Isoleucine	4.05	4.12	4.15	4.13	4.19	4.30	4.11^{b}	4.21^{a}	4.09	4.16	4.23	0.05	0.02	0.06	0.66
Leucine	10.71^{bc}	10.56^{bc}	10.37^{c}	10.90^{b}	11.40^{a}	11.48^{a}	10.55	11.26	10.80	10.98	10.93	0.10	<0.01	0.22	<0.01
Lysine	4.48^{a}	4.65^{a}	4.56^{a}	4.59^{a}	4.13^{b}	3.85^{b}	4.56	4.19	4.53	4.39	4.20	0.07	<0.01	<0.01	<0.01
Methionine	2.16	2.11	2.05	2.13	2.15	2.12	2.11	2.13	2.15	2.13	2.08	0.03	0.28	0.10	0.19
Phenylalanine	5.23^{ab}	5.28^{ab}	5.22^{ab}	5.17^{b}	5.25^{ab}	5.35^{a}	5.24	5.25	5.20	5.26	5.29	0.03	0.63	0.07	0.03
Threonine	4.35	4.32	4.43	4.33	4.16	4.24	4.37^{a}	4.25^{b}	4.34	4.24	4.33	0.05	<0.01	0.07	0.16
Tryptophan	0.25	0.40	0.63	0.65	0.66	0.72	0.43^{b}	0.67^{b}	0.45	0.53	0.67	0.09	<0.01	0.07	0.28
Valine	5.69	5.77	5.79	5.78	5.84	5.91	5.75	5.84	5.74	5.81	5.85	0.06	0.06	0.18	0.92
Sum of EAA, % of DM	2.71	2.69	2.82	2.69	2.60	2.60	2.74^{a}	2.63^{b}	2.70	2.65	2.71	0.01	<0.01	0.32	0.10
NEAA															
Alanine	8.31^{c}	8.32^{c}	8.30^{c}	8.63^{b}	8.68^{ab}	8.81^{a}	8.31	8.71	8.47	8.50	8.56	0.04	<0.01	0.08	0.04
Aspartic acid	8.24	8.31	8.49	8.05	7.73	7.90	8.35^{a}	7.89^{b}	8.14	8.02	8.19	0.09	<0.01	0.18	0.06
Cysteine	1.64	1.68	1.64	1.68	1.75	1.70	1.65	1.71	1.66	1.72	1.67	0.04	0.08	0.28	0.95
Glutamic acid	14.67^{a}	14.42^{a}	14.40^{a}	12.63^{c}	13.31^{b}	13.38^{b}	14.50	13.11	13.65	13.86	13.89	0.18	<0.01	0.37	0.01
Glycine	5.10	5.18	5.19	5.11	4.92	4.99	5.16^{a}	5.01^{b}	5.10	5.05	5.09	0.06	<0.01	0.68	0.11
Hydroxyproline	0.59	0.53	0.50	0.52	0.36	0.42	0.54^{a}	0.44^{b}	0.55^{a}	0.45^{b}	0.46^{b}	0.03	<0.01	<0.01	0.37
Hydroxylysine	1.14^{c}	1.15^{c}	1.13^{c}	3.33^{b}	3.44^{b}	3.82^{a}	1.14	3.53	2.24	2.30	2.48	0.06	<0.01	<0.01	<0.01
Ornithine	0.16^{d}	0.17^{d}	0.16^{d}	1.52^{c}	1.85^{b}	2.12^{a}	0.16	1.83	0.84	1.01	1.14	0.03	<0.01	<0.01	<0.01
Proline	7.04	7.10	6.92	7.40	7.70	7.54	7.02^{b}	7.55^{a}	7.22	7.40	7.23	0.10	<0.01	0.18	0.42
Serine	4.52	4.48	4.59	4.27	4.13	4.21	4.53^{a}	4.20^{b}	4.39	4.31	4.40	0.08	<0.01	0.40	0.66
Taurine	0.98	0.93	0.97	0.84	0.96	0.95	0.96	0.92	0.91	0.94	0.96	0.04	0.22	0.55	0.13
Tyrosine	3.31^{a}	3.30^{a}	3.33^{a}	2.52^{b}	2.28^{c}	1.73^{d}	3.31	2.18	2.91	2.79	2.53	0.03	<0.01	<0.01	<0.01
Sum of NEAA, % of DM	3.40	3.37	3.54	3.50	3.46	3.53	3.44	3.50	3.45	3.42	3.54	0.05	0.15	0.06	0.48
Sum of total AA, % of DM	6.11	6.06	6.36	6.19	6.06	6.13	6.18	6.13	6.15	6.06	6.25	0.09	0.47	0.15	0.25

[a–d]Means within a row with different superscripts differ ($P \leq 0.05$).

[1]CT = control (no protease addition); E2 = treatment to yield 200 mg of a protease formulation/kg of wet forage; E4 = treatment to yield 2,000 mg of a protease formulation/kg of wet forage.

[2]Pro = effect of protease dose; day = effect of day of ensiling.

treated silage; this finding was also reported by Windle et al. (2014). These results may suggest that use of a microbial inoculant to enhance aerobic stability would be required to offset the problem of a higher amount of available substrate, but future research is warranted to confirm this. In studies with the production of ethanol from grains, the addition of proteases also has resulted in stimulating the fermentation of yeasts (Pérez-Carrillo and Serna-Saldívar, 2007; Vidal et al., 2009; Wang et al., 2009), probably because the treatment with enzyme liberated more available substrates for growth. Yeasts in silages are undesirable because the production of ethanol is associated with high losses of DM, and lactate-assimilating yeasts are the primary initiators of aerobic spoilage in silages (Pahlow et al., 2003). Importantly, in our study, lower concentrations of protease (20 and 200 mg/kg) did not stimulate the growth of yeasts or the accumulation of ethanol in corn silage.

The amount of protease activity supplied by the lowest dose that we used in the current study was insufficient to affect soluble protein or NH_3-N in silage. Thus, as expected, the lowest treatment of protease did not improve IVSD in silage. The 1,000 mg/kg dose of protease resulted in fresh forage with a higher IVSD than untreated fresh forage. We have observed similar trends in our studies (Windle et al., 2014) with high (but not low) doses of proteases. Although the concentrations of SP increased in silages for each incremental dose above the 20 mg/kg dose and NH_3-N increased with each additional dose above 200 mg/kg, improvements in IVSD reached a statistical maximum with the 200 mg/kg dose.

We also measured total AA in forages and silages to determine whether there were any substantial changes in AA due to ensiling and treatment with protease. Ensiling and treatment with protease had no effect on the concentrations of total NEAA and overall total AA. However, ensiling caused a slight decrease in total essential AA, but this was not affected by treatment with protease. Individually, there were some interactions between the day of ensiling and different doses of

Table 3. Fermentation profile of fresh corn plants (d 0; after treatment but before ensiling) and ensiled corn plants (d 45) that were treated with various doses of an exogenous acidic protease formulation

Item[1]	pH	WSC,[2] % of DM	Lactic acid, % of DM	Acetic acid, % of DM	Ethanol, % of DM	LAB,[3] log cfu/g	Yeasts, log cfu/g
d 0							
CT	5.61^{a}	7.46	ND[4]	ND	ND	6.96	5.59^{a}
E1	5.64^{a}	7.78	ND	ND	ND	6.97	5.19^{a}
E2	5.65^{a}	7.87	ND	ND	ND	7.01	5.60^{a}
E3	5.65^{a}	7.93	ND	ND	ND	6.99	5.29^{a}
E4	5.68^{a}	6.64	ND	ND	ND	7.03	5.37^{a}
d 45							
CT	3.71^{bcd}	0.67	5.24	0.84	0.47^{c}	7.52	3.86^{b}
E1	3.75^{b}	0.69	5.79	1.01	0.57^{bc}	7.78	4.74^{ab}
E2	3.74^{bc}	0.96	5.74	0.90	0.55^{bc}	7.48	4.65^{ab}
E3	3.67^{cd}	1.39	6.42	1.00	0.85^{b}	7.30	5.82^{a}
E4	3.66^{d}	1.65	6.52	1.04	1.38^{a}	7.53	5.44^{a}
Day means							
d 0	5.65	7.53^{a}				6.99^{b}	5.41
d 45	3.71	1.07^{b}				7.53^{a}	4.90
Treatment means							
CT	4.66	4.07				7.24	4.42
E1	4.70	4.24				7.38	4.97
E2	4.69	4.42				7.09	5.13
E3	4.66	4.66				7.15	5.55
E4	4.67	4.15				7.28	5.41
SEM	0.02	0.60	0.54	0.10	0.09	0.08	0.25
P-value[5]							
Pro	0.42	0.87	0.46	0.61	<0.01	0.11	<0.01
Day	<0.01	<0.01				<0.01	<0.01
Pro × day	0.04	0.43				0.06	<0.01

$^{a–d}$Means within a column with different superscripts differ ($P < 0.05$).

[1]CT = control (no protease addition); E1 = treatment to yield 20 mg of a protease formulation/kg of wet forage; E2 = treatment to yield 200 mg of a protease formulation/kg of wet forage; E3 = treatment to yield 1,000 mg of a protease formulation/kg of wet forage; E4 = treatment to yield 2,000 mg of a protease formulation/kg of wet forage.

[2]Water-soluble carbohydrates.

[3]Lactic acid bacteria.

[4]Not determined.

[5]Pro = effect of protease dose; day = effect of day of ensiling.

protease for the EAA lysine, arginine, leucine, and phenylalanine because they were similar among treatments in fresh forages but were affected by treatment with the protease in silages. The concentrations of lysine and arginine were lower in silages treated with protease, but the concentrations of leucine and phenylalanine were higher in silages with protease compared with untreated silages. Changes in concentrations of total AA have also been reported when forages are ensiled (Crosby et al., 2001), but there is indeed a change in their distribution (more free AA) due to ensiling (Bergen et al., 1974). We did not measure free AA in our study, but future studies with proteases should. Substantial decreases in arginine due to ensiling have been reported by others as well (Ashbell et al., 1983; Crosby et al., 2001). Concentrations of AA in our experiment, averaged across treatments for d 45, were very similar to the results of Haque et al. (2013), who also measured AA in corn silages.

In the current experiment, there was no effect of day of ensiling or different doses of protease on the concentrations of NDF. These results agree with the findings of Der Bedrosian et al. (2012), who reported that ensiling did not affect the level of NDF, and Young et al. (2012), who reported that treatment with protease also did not affect the concentration of NDF in corn silage. The digestibility of NDF was decreased with ensiling in the current study, which is similar to results reported by others (Cherney, et al., 2007; Der Bedrosian et al., 2012; Young et al., 2012) and likely reflects some solubilization of NDF during the ensiling process. Although Colombatto et al. (2003) and Eun and Beauchemin (2008) both reported positive relationships between protease activity and NDF digestion, those studies were conducted on alfalfa, and treatment with protease was added to substrate several hours before in vitro fermentation and not as silage additives, as in our study. Furthermore, their proteases were characterized as alkaline-serine proteases, which most likely had a pH optimum that was much higher than the protease used in the current experiment.

CONCLUSIONS

We confirmed that treatment of freshly harvested corn plants with an exogenous protease could result in a greater improvement in ruminal IVSD after a short time of ensiling relative to the development that occurred via natural proteolytic mechanisms. Specially, a dose of 200 mg of protease/kg of fresh forage appeared to be sufficient to obtain this effect. Importantly, this dose did not have any adverse effect on the numbers of yeasts or the concentration of ethanol in silage. Although small differences were found in the concentrations of some specific EAA due to treatment with protease, the concentrations of total EAA, total NEAA, and total AA were unaffected in silage. A low dose of protease can be effective in improving IVSD without having negative effects on other characteristics of corn silage that is fed to dairy cows.

ACKNOWLEDGMENTS

We thank Nicola Walker (AB Vista, Wiltshire, UK) for technical advice and AB Vista for partially funding this study. In addition, we thank Caitlyn Merrill, Mariele Agarussi, and Lilian Rosa (University of Delaware) for their help with this experiment. We also thank Scott Hopkins and the farm crew at the University of Delaware (Newark) for planting and harvesting the corn.

REFERENCES

Allen, M. S., R. J. Grant, W. P. Weiss, G. W. Roth, and J. F. Beck. 2003. Effects of endosperm type of corn grain on starch degradability by ruminal microbes in vitro. J. Dairy Sci. 86(Suppl. 1):61. (Abstr.)

Ashbell, G., H. H. Theune, and D. Sklan. 1983. Changes in the amino acid compounds of the whole-crop wheat during ensiling and after fermentation. J. Sci. Food Agric. 34:321–327.

AOAC International. 2006. Official Methods of Analysis. 18th ed. AOAC Int., Gaithersburg, MD.

Bergen, W. G., E. H. Cash, and H. E. Henderson. 1974. Changes in nitrogenous compounds of the whole corn plant during ensiling and subsequent effects on dry matter intake by sheep. J. Anim. Sci. 39:629–637.

Cherney, D. J. R., J. H. Cherney, and W. J. Cox. 2007. Forage quality differences of corn hybrids as influenced by ensiling. Forage Grazinglands 5. https://doi.org/10.1094/FG-2007-0918-01-RS.

Colombatto, D., D. P. Morgavi, A. F. Furtado, and K. A. Beauchemin. 2003. Screening of exogenous enzymes for ruminant diets: Relationship between biochemical characteristics and in vitro ruminal degradation. J. Anim. Sci. 81:2628–2638.

Crosby, S. A., J. Zmich, R. A. Patton, M. J. Stevenson, and R. T. Ward. 2001. Effect of ensiling, storage time, and inoculant use on amino acid composition of alfalfa silage preserved in silage bags. J. Dairy Sci. 84(Suppl. 1):425. (Abstr.)

DePeters, E. J., G. Getachew, J. G. Fadel, L. Corona, and R. A. Zinn. 2007. Influence of corn hybrid, protease, and methods of processing on in vitro gas production. Anim. Feed Sci. Technol. 135:157–175.

Der Bedrosian, M. C., K. E. Nestor, and L. Kung Jr. 2012. The effects of hybrid, maturity, and length of storage on the composition and nutritive value of corn silage. J. Dairy Sci. 95:5115–5126.

Eun, J.-S., and K. A. Beauchemin. 2005. Effects of a proteolytic feed enzyme on intake, digestion, ruminal fermentation, and milk production. J. Dairy Sci. 88:2140–2153.

Eun, J.-S., and K. A. Beauchemin. 2008. Relationship between enzymatic activities and in vitro degradation of alfalfa hay and corn silage. Anim. Feed Sci. Technol. 145:53–67.

Ferraretto, L. F., P. M. Crump, and R. D. Shaver. 2015. Effect of ensiling time and exogenous protease addition to whole-plant corn silage of various hybrids, maturities, and chop lengths on nitrogen fractions and ruminal in vitro starch digestibility. J. Dairy Sci. 98:8869–8881.

Goering, H. K., and P. J. Van Soest. 1970. Forage Fiber Analyses. Apparatus, Reagents, Procedures and Some Applications. Agric. Handbook No. 379. USDA Agricultural Research Service, Washington, DC.

Haque, M. N., H. Rulquin, and S. Lemosquet. 2013. Milk protein responses in dairy cows to changes in postruminal supplies of arginine, isoleucine, and valine. J. Dairy Sci. 96:420–430.
Hall, M. B. 2009. Determination of starch, including maltooligosaccharides, in animal feeds: Comparison of methods and a method recommended for AOAC collaborative study. J. AOAC Int. 92:42–49.
Hallada, C. 2009. Why new corn silage doesn't feed as well. Hoard's Dairyman 154:712.
Hoffman, P. C., N. M. Esser, R. D. Shaver, W. K. Coblentz, M. P. Scott, A. L. Bodnar, R. J. Schmidt, and R. C. Charley. 2011. Influence of ensiling time and inoculation on alteration of the starch-protein matrix in high-moisture corn. J. Dairy Sci. 94:2465–2474.
Junges, D., G. Morais, M. H. F. Spoto, P. S. Santos, A. T. Adesogan, L. G. Nussio, and J. L. P. Daniel. 2017. Influence of various proteolytic sources during fermentation of reconstituted corn grain silages. J. Dairy Sci. 100:9048–9051.
Klingerman, C. M., W. Hu, E. E. McDonell, M. C. Der Bedrosian, and L. Kung Jr. 2009. An evaluation of exogenous enzymes with amylolytic activity for dairy cows. J. Dairy Sci. 92:1050–1059.
Krishnamoorthy, U., T. V. Muscato, C. J. Sniffen, and P. J. Van Soest. 1982. Nitrogen fractions in selected feedstuffs. J. Dairy Sci. 65:217–225.
Kung, L., Jr., R. J. Treacher, G. A. Nauman, A. M. Smagala, K. M. Endres, and M. A. Cohen. 2000. The effect of treating forages with fibrolytic enzymes on its nutritive value and lactation performance of dairy cows. J. Dairy Sci. 83:115–122.
McCarthy, M. M., M. A. Engstrom, E. Azem, and T. F. Gressley. 2013. The effect of an exogenous amylase on performance and total-tract digestibility in lactating dairy cows fed a high-byproduct diet. J. Dairy Sci. 96:3075–3084.
Muck, R. E., and J. T. Dickerson. 1988. Storage temperature effects on proteolysis in alfalfa silage. Trans. ASAE 31:1005–1009.
Nelson, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375–380.
Okuda, H., S. Fuji, and Y. Kawashima. 1965. A direct colorimetric method for blood ammonia. Tokushima J. Exp. Med. 12:11–23.
Pahlow, G., R. E. Muck, F. Driehuis, S. J. W. H. Oude Elferink, and S. F. Spoelstra. 2003. Microbiology of ensiling. Pages 31–93 in Silage Science and Technology. D. R. Buxton, R. E. Muck, and J. H. Harrison, ed. American Society of Agronomy, Madison, WI.
Pérez-Carrillo, E., and S. O. Serna-Saldívar. 2007. Effect of protease treatment before hydrolysis with α-amylase on the rate of starch and protein hydrolysis of maize, whole sorghum, and decorticated sorghum. Cereal Chem. 84:607–617.
Philippeau, C., and B. Michalet-Doreau. 1998. Influence of genotype and ensiling of corn grain on in situ degradation of starch in the rumen. J. Dairy Sci. 81:2178–2184.
Sheperd, A. C., and L. Kung Jr. 1996. Effects of an enzyme additive on composition of corn silage ensiled at various stages of maturity. J. Dairy Sci. 79:1767–1773.
Snedecor, G. W., and W. G. Cochran. 1980. Statistical Methods. 6th ed. Iowa State Univ. Press, Ames.
Sumantha, A., C. Larroche, and A. Pandley. 2006. Microbiology and industrial biotechnology of food-grade proteases: A perspective. Food Technol. Biotechnol. 44:211–220.
Tilley, J. M. A., and R. A. Terry. 1963. A two-stage technique for the in vitro digestion of forage crops. J. Br. Grassl. Soc. 18:104–111.
Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583–3597.
Vidal, B. C., Jr., K. D. Rausch, M. E. Tumbleson, and V. Singh. 2009. Protease treatment to improve ethanol fermentation in modified dry grind corn processes. Cereal Chem. 86:323–328.
Wang, P., D. B. Johnson, K. D. Rausch, S. J. Schmidt, M. E. Tumbleson, and V. Singh. 2009. Effects of protease and urea on a granular starch hydrolyzing process for corn ethanol production. Cereal Chem. 86:319–322.
Ward, R. T., and M. B. de Ondarza. 2008. Effect of month of sample submittal on corn silage nutrient fractions, starch availability, NDF digestibility, and fermentation profiles measured at a commercial forage-testing laboratory. J. Dairy Sci. 91(Suppl. 1):30. (Abstr.)
Windle, M. C., N. Walker, and L. Kung Jr. 2014. Effects of an exogenous protease on the fermentation and nutritive value of corn silage harvested at different dry matter contents and ensiled for various lengths of time. J. Dairy Sci. 97:3053–3060.
Young, K. M., J. M. Lim, M. C. Der Bedrosian, and L. Kung Jr. 2012. Effect of exogenous protease enzymes on the fermentation and nutritive value of corn silage. J. Dairy Sci. 95:6687–6694.