

5-1-2012

Assessments of an Exogenous Proteolytic Enzyme in Beef Steer Diets to Improve Growth Performance and Ruminant fermentation

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Recommended Citation

Vera, Juan Manuel, "Assessments of an Exogenous Proteolytic Enzyme in Beef Steer Diets to Improve Growth Performance and Ruminant fermentation" (2012). *All Graduate Theses and Dissertations*. Paper 1351.
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ASSESSMENT OF AN EXOGENOUS PROTEOLYTIC ENZYME IN BEEF STEER
DIETS TO IMPROVE GROWTH PERFORMANCE AND RUMINAL
FERMENTATION

by

Juan Manuel Vera

A thesis submitted in partial fulfillment
of requirements for the degree

of

MASTER OF SCIENCE

in

Animal Science

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2012

ABSTRACT

Assessments of an Exogenous Proteolytic Enzyme in Beef Steer Diets to Improve
Growth Performance and Ruminal fermentation

by

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Utah State University, 2011

Major Professor: Jong-Su Eun

Department: Animal, Dairy, and Veterinary Sciences

A series of experiments were conducted to investigate the effects of adding an exogenous proteolytic enzyme (EPE) on the growth performance of beef steers fed growing and finishing diets containing 30% dried distillers grains with solubles (DDGS; Exp. 1), and results corroborated by in vitro ruminal fermentation in continuous cultures (Exp. 2). In Exp. 1, 48 group-penned Angus crossbred steers were randomly assigned to 2 treatments ($n = 6$) in a completely randomized design: DDGS TMR (DT) without and with EPE (27 mg of azocasein hydrolyzed/min/kg DM TMR). The addition of EPE during the growing phase increased DMI ($P = 0.02$), but had no effects on final BW, BW change, ADG, and G:F. Adding EPE during the growing phase decreased NDF digestibility, whereas the digestibility of DM, CP, and ADF were not affected. There was

a tendency for both ADG ($P = 0.09$) and final BW ($P = 0.11$) to increase during the finishing phase without affecting BW change and G:F. As opposed to the growing phase, EPE increased digestibility ($P < 0.04$) of DM, CP, NDF, and ADF. In Exp. 2, 4 dietary treatments were assessed in continuous cultures; non-DDGS TMR (NDT) or DT finishing beef steer diet was combined without or with EPE in a 2×2 factorial design. The DT was the same diet used as the finishing diet in Exp. 1, and dose rate of EPE was the same as Exp. 1. Feeding the DT increased total VFA concentration ($P = 0.01$) which corresponded with a decreased ($P < 0.01$) pH compared with the NDT diet (5.8 vs. 6.0) regardless of EPE supplementation. Supplementing EPE tended to increase ($P = 0.07$) the total VFA concentration in both diets, but only increased digestibility of DM, OM, and NDF when added to the DT diet ($P < 0.05$), leading to tendencies on TMR \times enzyme interaction ($P < 0.10$). Addition of the EPE product assessed in this study resulted in positive responses in Exp. 1 and 2 when added to finishing beef steer diets, and thus it is clear that use of protease enzyme products may be more effective in high concentrate diets such as finishing beef steer diets containing DDGS.

(68 pages)

PUBLIC ABSTRACT

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A series of experiments were conducted to investigate the effects of adding an exogenous proteolytic enzyme (**EPE**) on growth performance and ruminal fermentation characteristics of beef steers fed growing and finishing diets containing 30% dried distillers grains with solubles (**DDGS**).

In the first experiment, 48 beef steers were divided into two treatments: DDGS diets with and without EPE. Dry matter intake (**DMI**) was increased ($P = 0.02$) by the addition of EPE, but neutral detergent fiber (**NDF**) digestibility was decreased on the growing phase. For the finishing phase, average daily gain (**ADG**) and final body weight (**BW**) tended to increase ($P = 0.09, 0.11$ respectively) and digestibilities of dry matter (**DM**), crude protein (**CP**), NDF and ADF also increased ($P = 0.04$).

An in vitro experiment was then performed to corroborate results of the in vivo experiment. Diets containing no DDGS (**NDT**) were added to analyze effects of DDGS and its interaction with EPE. Feeding the DT increased total VFA concentration ($P = 0.01$) which corresponded with a decreased ($P < 0.01$) pH compared with the NDT diet (5.8 vs. 6.0) regardless of EPE supplementation. Supplementing EPE tended to increase ($P = 0.07$) the total VFA concentration in both diets, but only increased digestibility of DM, OM, and NDF when added to the DT diet ($P < 0.05$), leading to tendencies on TMR \times enzyme interaction ($P < 0.10$).

Addition of the EPE product assessed in this study resulted in positive responses in Exp. 1 and 2 when added to finishing beef steer diets, and thus it is clear that use of protease enzyme products may be more effective in high concentrate diets such as finishing beef steer diets containing DDGS.

ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Jong-Su Eun, for his patience, wisdom, and guidance. I especially appreciate all the hours dedicated for posters, manuscripts, and presentations. Your enthusiasm and dedication to teach animal nutrition is to be admired and emulated.

I would also like to thank Dr. Dale ZoBell and Dr. Allen Young for serving in my committee. Your expertise was essential in understanding aspects of my research that I had no clue about. Thanks for lifting my spirits whenever I needed and thanks to Dr. ZoBell for the Saturday fishing. It was a great way for me to recharge energies and clear my mind for a while.

I gratefully acknowledge the Research Assistantship Program provided by Danisco Animal Nutrition (Marlborough, UK) and Utah Agricultural Experiment Station, Utah State University.

I express my deepest gratitude for my Skaggs Lab family, Novi, Chris, James, Christina, Katie, Shane, Sheldon, and Fernando, for always being there through thick and thin and for helping me in any way I needed, whether it was an extra hand for sampling or a shoulder to cry on. All of you were essential for me to complete my program, and I will never forget any of you. In addition, I thank E. Galloway and other Utah State University Beef Research Unit staff for technical assistance.

Finally and most importantly, I would like to thank my family. Pedro, your emotional support has kept me going from day one. Maria Del Mar, every time I would talk to you over webcam or on the phone you managed to bring a smile to my face even when I was having the worst day possible. Veronica, you and I may not share the same blood, but we

might as well. Thank you for everything, it would take me a whole new thesis to express how grateful I am for everything you've done for me. Mom, thank you for doing the little things that mean the most in life for a son, like making sure I was eating and sleeping enough, sending me a piece of home even when I was miles away, and always supporting me in any way possible. Dad, "mi querido Viejo," thank you for inspiring me to persevere, to keep going no matter how grim things look, to never give up, and to always say "thank you". In my eyes you are always the wisest man I have ever known and I can only hope to follow your steps and be one day at least half the man you are.

Juan M. Vera

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LIST OF ABBREVIATIONS

A:P = acetate-to-propionate ratio

ADF = acid detergent fiber

ADG = average daily gain

AIA = acid insoluble ash

BW = body weight

CDS = condensed distillers solubles

CH₄ = methane

CP = crude protein

DDG = dried distillers grains

DDGS= DDG with solubles

DM = dry matter

DMI = DM intake

DT = DDGS-based total mixed ration

DT-EPE = DDGS total mixed ration without exogenous proteolytic enzyme

DT+EPE = DDGS total mixed ration with exogenous proteolytic enzyme

EPE = exogenous proteolytic enzyme

FA = fatty acids

G:F = gain-to-feed ratio

HCW = hot carcass weight

KPH = kidney, pelvic, and heart fat

LM = longissimus muscle

NFC = nonfibrous carbohydrate

NDF = neutral detergent fiber

NDT = non DDGS-based total mixed ration

NH₃-N = ammonia-nitrogen

NE_L = net energy for lactation

OM = organic matter

RDP = rumen degradable protein

SEM = standard error of least square means

TMR = total mixed ration

VFA = volatile fatty acids

WDG = wet distillers grains

WDGS= WDG with solubles

INTRODUCTION

In order for feed enzyme technology to be applied on-farm, many factors must be considered such as product formulation and dose rate, as well as key enzymatic activities and enzyme/substrate specificity, so that feed enzyme supplementation in ruminant diets can ensure consistent results on the efficacy of feed enzyme products in cattle diets and resultant animal performance (Beauchemin et al., 2003). For example, various studies have been done to examine the effects of enzyme supplementation on growth performance of feedlot cattle in both high-forage and high-concentrate diets (Beauchemin et al., 1995; ZoBell et al., 2000); however, results of supplementing feed enzymes have been more consistent for high-grain diets compared with those of high-forage diets. Also, most studies have been focused on the efficacy of fibrolytic enzymes that contain mainly endoglucanase, xylanase, and exoglucanase activities, whereas other activities such as protease have not been extensively researched. Recent evidence 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, 2005a), suggesting a high potential for supplementing exogenous proteolytic enzyme (**EPE**) product to improve nutrient utilization and growth performance of beef steers.

There is increasing interest in determining how best to use dried distillers grains with solubles (**DDGS**) as the quantities available increase. Because DDGS contains large amounts of digestible fiber, there is a high potential to increase its digestibility by use of feed enzymes which increase the rate of fiber digestion in the rumen by increasing the hydrolytic capacity within the ruminal environment. The objective of this study was to examine the effects of supplementing an EPE product in growing and finishing diets on

growth performance and carcass characteristics of beef steers. In addition, *in vitro* ruminal fermentation was assessed using continuous cultures to explore ruminal microbial metabolism in response to EPE supplementation in DDGS-containing finishing beef steer TMR.

REVIEW OF LITERATURE

Use of Distillers Grains with Solubles in Feedlot Diets

With the expansion of the ethanol industry there is an increase in the production of by-products. One of the by-products is dried distillers grains with solubles (**DDGS**) coming from fermentation of cereal grains to produce beverage alcohol and in more recent years as a source of renewable energy. This process involves the fermentation of starch found in ground grain to produce alcohol. The fermented mash is then processed further to remove the alcohol and the water associated with the DM. Distillation of the mash then produces feed slurry called spent stillage. The coarse particles from the stillage are removed, and this can be sold as either wet distillers grains (**WDG**) or dried and sold as dried distillers grains (**DDG**). The remaining liquid fraction after the distillation process is called thin stillage, which contains grain particles and yeast cells, and can be evaporated to produce condensed distillers solubles (**CDS**). The CDS can then be dried along with WDG or DDG to produce wet distillers grains with solubles (**WDGS**) or **DDGS**. Because of the processing of corn to ferment ethanol for biofuel, starch is mostly removed, and the other nutrients associated with the grains become more concentrated. Protein, for example, increases from approximately 9% in the original corn grain to 27% in the whole stillage (Stock et al., 2000), whereas NDF increases from 9% to 46%, and fat increases from 4.3% to 10.3% on DM basis (NRC, 1996). These concentrations can also vary between ethanol plants because of the different heating treatments used between processing plants.

WDGS vs. DDGS

Feeding WDGS avoids the cost of drying the product, but WDGS contain about 70% moisture, which limits their transport to shorter distances and makes storage more challenging. During winter, WDGS may freeze into clumps, leading to inconsistency of the ration due to the poor blending capacity of the frozen WDGS. During warmer months, WDGS tend to mold, and have a short shelf life of about 7 d (Kent and Wright, 2002). In a study by Ham et al. (1994) the WDGS contained 47% greater feeding value than corn, and DDGS exerted 24% greater feeding value. The drying process appears to reduce the energy value of the DDGS. The authors noted that because DM was determined by oven drying, it is conceivable that some volatile organic matter was lost and contributed to this difference (Ham et al., 1994). Although WDGS has a higher feeding value than DDGS, both have considerably higher feeding values than corn, and thus considerations regarding handling, transport, and cost play an important role in deciding which DGS to feed. Since DDGS only contain 10-12% moisture, they can be shipped greater distances more economically and conveniently than WDGS. In addition, DDGS can be easily mixed with other ingredients, and are easy to store. Although DDGS have high levels of fat, rancidity during summer months is usually not a concern (Kent and Wright, 2002; Schingoethe, 2006).

Levels of Distillers Grains in Feedlot Diets

There is an increasing interest in determining how to best use DGS as the quantities available increase. Optimal inclusion rate of DGS can vary greatly depending upon their nutrient contents. Daubert et al. (2005) evaluated levels of WDGS (0, 8, 16, 24, 32, and

40%) in steam flaked corn-based diets fed to feedlot heifers. They found that DMI decreased linearly with the addition of WDGS, while ADG and G:F quadratically improved, peaking at 8 and 16% WDGS, respectively. Vander Pol et al. (2006) evaluated levels of 0, 10, 20, 30, 40, and 50% WDGS in beef finishing diets replacing a portion of corn grain. They also observed a quadratic response in ADG and G:F in response to WDGS level with the optimal level being at 20%. In addition, they found that feed efficiency was greater for all levels of WDGS compared with 0% WDGS diet.

A similar trend was shown with DDGS, as Buckner et al. (2008) observed a quadratic response in G:F when cattle were fed levels of 0, 10, 20, 30, and 40% DDGS. A meta-analysis completed by Klopfenstein et al. (2008) showed a quadratic response in ADG and a cubic response in G:F, as level of DDGS in diet increased from 0 to 40%. They noted that the maximum ADG was between 20 and 30%, and maximum G:F was observed between 10 and 20% DDGS (Klopfenstein et al., 2008). The study also evaluated the differences between WDGS and DDGS, and showed that inclusion levels for maximum ADG and G:F was lower for DDGS than for WDGS (Klopfenstein et al., 2008).

Leupp et al. (2009a) performed a study on increasing levels of DDGS in finishing diets with 70% concentrate. Treatments consisted of increasing DDGS at 0, 15, 30, 45, or 60% of diet DM replacing a combination of dry rolled corn, sunflower meal, and urea. The authors found a quadratic response on OM intake; greater OM intake was achieved at 15% DDGS, but lowest intake at 60% DDGS. Intake of CP also tended ($P = 0.08$) to be quadratic with 0% DDGS having the least intake and 45% DDGS having the most. The lower responses at higher DDGS inclusion rates could be due to the fact that DDGS

contains higher levels of sulfur. According to NRC (1996), the maximum tolerable concentration of sulfur has been estimated at 0.4% of diet DM. Uwituze et al. (2011) reported that increasing levels of sulfur (0.65% DM) decreased DMI and ADG by 8.9% and 12.9%, respectively, but had no effects on G:F. Cattle fed high sulfur diets also yielded 4.3% lighter HCW and had 16.2% less KPH than steers fed the moderate sulfur diet (Uwituze et al., 2011). Thus, as DDGS inclusion level increases, sulfur content in the diet could potentially increase to threshold levels which could explain the quadratic responses shown in most studies.

Ruminal Metabolism and Digestibility of Distillers Grains

A number of metabolism studies have been conducted in an effort to investigate effects of DGS on ruminal metabolism and digestibility. Leupp et al. (2009a) performed a study using cannulated steers to assess ruminal fermentation and digestion, and the authors found that as dietary DDGS increased, ruminal pH increased. This can be the result of decreased starch levels in the DDGS compared to corn grain. Feeding DDGS could conceivably be beneficial in high concentrate diets, because starch is extracted during fermentation process, and consequently fiber content is increased. However, when Corrigan et al. (2008) fed 0 or 40% WDGS in dry rolled corn-, high moisture corn-, or steam flaked corn-based diets, their results showed that feeding WDGS did not increase ruminal pH. Similarly, in a study by May et al. (2009), ruminal pH was lower in cattle fed 25% DDGS compared to those fed 0% DDGS as a partial replacement of steam flaked corn or dry rolled corn in finishing diets. Therefore, effects of feeding DDGS on ruminal pH may have been affected by dietary inclusion rate of WDGS and DDGS,

processing method of basic grain ingredients, and their potential interactions during ruminal fermentation.

Vander Pol et al. (2009) conducted a metabolism study to determine the effect of feeding WDGS or supplemental fat on performance and rumen metabolism and digestibility, with an aim of determining whether fat is responsible for the greater energy value of WDGS compared with corn in finishing diets. Dietary treatments were: 0% corn oil, 2.5% corn oil, 5.0% corn oil, 0% WDGS, 20% WDGS, or 40% WDGS. Overall results of the study suggest that WDGS did not control ruminal acidosis by increasing ruminal pH; rather, the high energy value of WDGS was due to higher fat digestibility, more propionate production, and more unsaturated fatty acids reaching the duodenum. May et al. (2009) found increased propionate when adding DDGS to steam flake corn- and dry rolled corn-based diets. In the study by Vander Pol et al. (2009) the digestibility of added fat as corn oil was 70%, while fat added as WDGS was digested at 81%. Steers fed WDGS had 21% higher unsaturated fatty acids flowing in duodenum than their counterparts fed similar amounts of corn oil. Poor digestion of saturated fats could explain this negative influence.

According to Plascencia et al. (2003), intestinal fatty acid digestion decreased with level of total fatty acid intake, regardless of degrees of saturation. Feeding DDGS in dry rolled corn- or steam flaked corn-based finishing diets resulted in lower total tract digestibility of ether extracts compared with feeding no DDGS (May, 2007). The negative effect of fat on ruminal digestion has been well demonstrated, and thus feeding DDGS may exert negative impacts on nutrient digestion and ruminal fermentation, depending upon its inclusion rate.

May et al. (2009) evaluated the digestibility of DDGS in diets comprised of steam flaked corn or dry rolled corn. Cattle consuming DDGS tended ($P = 0.10$) to have lower apparent total tract digestibility of DM and OM compared to those without DDGS in either grain processing method. Likewise, in a study by Depenbusch et al. (2007), digestibility of DM and OM was decreased by adding approximately 13% DDGS or degermed corn DDGS to steam flaked corn diets.

Feeding DDGS in dry rolled corn-based diets resulted in a lower magnitude of change in digestibility compared with feeding DDGS in steam flaked corn-based diets (May et al., 2009). This is because steam flaked corn has a higher digestibility than dry rolled corn because of processing which in turn contributes to the greater magnitude of change seen on steam flaked corn-based diets with added DDGS. When DDGS was added to a 70% concentrate diet, no differences were seen on total tract OM digestibility; however, ruminal OM digestibility decreased linearly as inclusion rate of DDGS increased (Leupp et al., 2009a). The authors attributed this result to a shift in the site of digestion of DDGS due to a faster passage rate of DDGS in the rumen. This shift in site of digestion from the rumen to the small intestine could also be due to the increased fat content when increasing the inclusion rate of DDGS.

Eun et al. (2009b) found no changes in DM digestibility or VFA profiles when DDGS was used to replace barley grain as a concentrate source on beef cattle diets. However, for a moderate grass forage diet, supplementation of DDGS increased OM digestibility, but acetate concentration and acetate-to-propionate ratio followed a similar linear decrease to that of Leupp et al. (2009a) with propionate concentration showing a quadratic response (Leupp et al., 2009b). This could imply that DDGS has a higher digestibility than

medium quality forages, because it is less lignified, but is of lower digestibility than processed corn due to the higher fiber content and the lack of starch. In this same study fiber digestibility followed a linear increase with increasing DDGS inclusion (Leupp et al., 2009b), but when DDGS was added to a 70% concentrate diet, NDF digestibility followed a quadratic response as inclusion rate increased (Leupp et al., 2009a). For moderate quality forage the linear response is expected because of lower fiber digestibility in the forage due to lignification. By adding DDGS, more digestible fiber was added, increasing overall fiber digestibility.

For high concentrate diets, total tract digestibility of CP was increased either linearly for higher DDGS inclusion rates (Leupp et al., 2009a) or to be higher when compared with corn as an energy source under limited feeding on beef steers (Felix et al., 2011). For DDGS used as a supplement on moderate quality forage diets, CP digestibility followed a quadratic response with the highest digestibility being at 1.2% of BW inclusion rate (DM basis; Leupp et al., 2009b). However, Uwituze et al. (2010) found decreases in final BW, ADG, and G:F, but no changes when those results were adjusted with carcass. These results indicate that energy digestibility between treatments would not differ. One reason postulated for the non-response was that when DDGS was added to the diet, it replaced a portion of the urea making N availability a limiting factor for microbial growth and subsequent digestion of substrates. Dried distillers grains contain less than 30% RDP (NRC, 1996).

Carcass Characteristics of Cattle Fed Distillers Grains

Carcass characteristics are directly related to how well an animal performs, and therefore DGS not only affects feedlot performance, but it also alters carcass characteristics. There has been many studies showing differences in carcass characteristics when DDGS is added to the diet. May et al. (2009) observed that feeding 25% DDGS increased dressing percentage compared to cattle fed 0% DDGS. Depenbusch et al. (2008) observed decreases in HCW and LM area when cattle were fed 25% WDGS in steam flaked corn-based diets. Daubert et al. (2005) evaluated levels of WDGS (0, 8, 16, 24, 32, and 40%) and observed that LM area linearly decreased, while yield grade linearly increased. When feeding varying levels of WDGS (0, 15, 27.5, and 40%) in finishing diets, Corrigan et al. (2007) observed that HCW and 12th rib fat thickness responded quadratically to the inclusion of WDGS. Al-Suwaiegh et al. (2002) evaluated the effect of feeding corn or sorghum grain distillers grains at 30% of the diet DM compared with dry rolled corn-based diet without the distillers grains (control). They observed that the addition of distillers grains increased HCW, 12th rib fat thickness, and yield grade compared with the control diet, but had no effect on marbling score. A meta-analysis using 21 individual feeding studies from 6 U.S. states to evaluate carcass fat distribution of feedlot cattle fed various levels of distillers grains was completed by Reinhardt et al. (2007). They observed that feeding low levels of distillers grains (16% and lower) increased marbling score, while high levels of distillers grains (33% or higher) depressed marbling score. Feeding moderate levels of distillers grains (23%) resulted in high marbling scores. However, concern was raised on the change in overall body fatness (measured as yield grade), as it was even more dramatic than changes in marbling score

in cattle fed distillers grains (Reinhardt et al., 2007). Distillers grains typically increase HCW, 12th rib fat thickness, and yield grade, but it decreases LM area, indicating that feeding DGS can induce heavier carcasses with more fat deposition as back fat instead of marbling. Differences in carcass characteristics when adding DDGS to beef steer diets could be due to changes in metabolism of lipid due to DDGS, influencing meat composition (Eun et al., 2009b). Also, changes in VFA profiles can affect carcass traits in beef cattle. Furthermore, lowered RDP of dietary CP could also cause shifts in ruminal fermentation that can affect carcass characteristics. In addition, total energy intake affects partitioning of fat deposition in the growing and finishing phases, although it is more pronounced in the growing phase (Schoonmaker et al., 2003).

Use of Feed Enzymes on Beef Cattle Production

Researchers in the 1960s were first to examine the use of oxogenous enzymes in ruminant animals (Burroughs et al., 1960; Rovics and Ely, 1962; Rust et al., 1965). However, these responses were highly variable, mainly because the mode of action was not well understood, but also because enzyme preparations were expensive at the time which reduced the interest in exogenous enzyme research. Reductions in fermentation costs together with increases in feed costs, concern over the use of antibiotic feed additives, and development of new enzyme products have renewed interest in exogenous enzyme research (McAllister et al., 2001; Beauchemin et al., 2008).

Enzyme additives are concentrated fermentation products that contain specific enzyme activities and are used to catalyze reactions by which feedstuffs are digested into their chemical components. These products have a great potential to increase fiber

degradation in cattle which, in turn, could enhance feed utilization and animal performance. The main purpose of using enzyme additives is to increase fiber digestion in the rumen, because even under ideal conditions, total tract digestibility of NDF in cattle is usually less than 50%. The effectiveness of enzyme additives is still mostly variable, and these inconsistencies have been attributed to differences in product formulation, dose rates, composition of the targeted forage, and method of providing enzyme additives (Beauchemin et al., 2003). The key activities needed for forage degradation can vary between forages due to differences in chemical composition of the targeted forage. In spite of these challenging conditions, enzyme additives have shown great potential to increase meat production, animal performance, and feed digestion (McAllister et al., 2001; Beauchemin et al., 2003; Krause et al., 2003). For example, Eun and Beauchemin (2008) performed a meta-analysis study using data from in vitro studies performed with alfalfa hay and corn silage. Added endoglucanase, exoglucanase, xylanase, and protease activities, in addition to endoglucanase:xylanase ratio, were included in the analyses. Based on their results, the authors concluded that exoglucanase activity was the main activity needed to improve in vitro NDF degradation of both alfalfa hay and corn silage. They also reported that type and characteristics of xylanases may be more important than added xylanase activity. Overall they concluded that there is a high potential to increase forage digestibility by ruminants using enzyme additives that are formulated to supply appropriate enzymatic activities (Eun and Beauchemin, 2008). Thus, enzyme additives that are carefully tailored to provide the key enzymatic activities needed to degrade a specific substrate at an optimum dose rate could potentially increase

production performance of beef cattle by increasing fiber digestibility and altering ruminal fermentation characteristics.

Main Enzymes Involved in Degradation of Plant Fiber

Main Enzymatic Activities

Most of the enzyme preparations are marketed on their capacity to degrade plant cell walls and are often referred to as cellulases, hemicellulases, or xylanases, because cellulose and hemicellulose are the major structural polysaccharides in plants (Van Soest, 1994). However, none of these commercial products are preparations of a single enzymatic activity; secondary enzyme activities such as amylases, proteases or pectinases are present. The types of cellulases and hemicellulases can vary greatly depending upon the microbial strain, substrate used, and culture conditions (Gashe, 1992). The degradation of cellulose and hemicellulose requires an array of different enzymes working together, and differences in the proportions and activities of these enzymes impact the efficacy of cell wall degradation by the marketed products.

To digest cellulose, there are 3 main enzymes involved: endoglucanase, exoglucanase, and β -glucosidase. Generally speaking, endoglucanase attacks the cellulose molecule at random spots to produce oligomers of varying degrees of polymerization. After this, exoglucanase attacks the oligomers at the non-reducing ends producing cellobiose. This cellobiose is then hydrolyzed by β -glucosidases releasing glucose. Therefore, a range of cellulase enzymes are needed to hydrolyze native cellulose, since both endoglucanase and exoglucanase act synergistically to hydrolyze the

main molecule of cellulose, releasing cellobiose so that β -glucosidase can then act on the cellobiose releasing glucose.

For the degradation of the xylan core polymer to release soluble sugars, a different array of enzymes is needed. Xylanase and β -1,4-xylosidase will yield xylooligomers and xylose, respectively (Bhat and Hazlewood, 2001). For the digestion of the side chains from hemicellulose, β -mannosidase, α -L-arabinofuranosidase, α -D-glucuronidase, α -D-galactosidase, acetyl-xylan esterase, and ferulic acid esterase are all needed (White et al., 1993; Bhat and Hazlewood, 2001).

Proteases

Proteases are other enzymes that could represent opportunities of ruminant feed enzyme products. Two in vitro studies (Colombatto et al., 2003a,b) reported large increases in in vitro NDF degradability of alfalfa hay and a total mixed ration as a result of supplementation with an enzyme product containing protease activity (Protex 6L[®], Genencor International, Rochester, NY), but no cellulase or xylanase activity. Contrary to general expectation, protein degradation was only numerically increased. In a follow-up study done by Eun and Beauchemin (2005a), that particular exogenous proteolytic enzyme (EPE) product was fed to dairy cows using a dose rate (1.25 mL/kg diet DM) similar to that used in vitro (Colombatto et al., 2003a). Supplementation of a low forage diet (18.2% barley silage, 16.0% alfalfa hay, and 65.8% concentrate on DM basis) with EPE increased total tract NDF digestibility by 26%, but there was no effect on NDF digestibility of a high forage diet (44.5% barley silage, 16.0% alfalfa hay, and 39.5% concentrate on DM basis). The lack of effect on the high forage ration could have been due to the higher concentration of barley silage in this diet, since this product has been

shown to be ineffective for barley silage (McGinn et al., 2004). When a different EPE product was used (Papain, Dyadic International, Inc., Jupiter, FL), in vitro NDF degradability of alfalfa hay was improved by 19% using a dose of 0.25 mg/g DM, and NDF degradability of corn silage was improved by 17% using a dose of 0.5 mg/g DM (Eun and Beauchemin, 2007).

It is then clear that protease activity could play a significant role in fiber degradation of some forages. A possible mechanism by which proteases enhance fiber degradation is through the attack of some of the cell wall N containing components that act as physical barriers to degradation (Colombatto et al., 2003b). For example, Jung (1997) suggested that tyrosine residues could play a role in the cross-linking of dicotyledonous plants. In addition to protease activity, the type of protease should also be taken into consideration. Eun et al. (2007) tested 2 protease additives in vitro and observed that an alkaline protease increased fiber degradability of alfalfa hay, whereas an acidic protease had no effect. The relationship between the protease activity and the improvement in fiber degradation could then be dependent on the type of protease.

Like fibrolytic enzymes, the efficacy of the protease also depends on the type of target forage. Eun and Beauchemin (2005b) reported that although protease improved the in vitro degradation of alfalfa hay and barley silage, protease was more effective for the alfalfa hay. Different responses to protease among forages were also reported previously by Colombatto et al. (2003b) who concluded that the same protease product was effective when used with alfalfa hay, but not with corn silage. McGinn et al. (2004) reported no effect of this product on total tract digestibility and DM intake when fed to beef cattle receiving a diet containing 75% barley silage (DM basis). Furthermore, Eun and

Beauchemin (2005b) reported that adding protease improved in vitro degradation of alfalfa hay, but not alfalfa silage. Although it is not clear why the protease is not effective for silages, it could be due to the higher quality of silages or fermentation acids that are produced during the ensiling process. Additional research is needed on the mode of action of proteases to elucidate the increase on dry forage digestibility, but not on ensiled forage.

Mode of Action on Feed Enzymes

It has been shown that exogenous enzymes are most effective when applied to the feed prior to its ingestion by cattle (Beauchemin et al., 2003). There is evidence that applying fibrolytic enzymes to feed prior to feeding alters the structure of the feed, making it more amenable to degradation. Nsereko et al. (2000) applied an enzyme product to alfalfa hay that was then autoclaved to inactivate enzyme activities and washed to remove any product of hydrolysis. This eliminated the possibility of chemotactic mechanisms or synergy between microbial enzymes and exogenous enzymes. They found that in vitro NDF degradation was higher at 12 and 48 h for treated hay than un-treated hay, and that this effect was enhanced by a longer pre-incubation with enzymes. Thus, the effects of exogenous enzyme on digestion were observed in the absence of active enzymes and soluble hydrolysis products, suggesting that exogenous enzymes caused some structural changes to the plant fiber that improved digestion.

Another important reason to apply enzymes to the feed prior to ingestion is to enhance the binding of the enzyme to its substrate which in turn increases the resistance of the enzyme to the proteolytic activity in the rumen. When applied to the feed prior to

ingestion, enzymes are particularly stable, because the presence of the substrate is known to increase the resistance of the enzyme to proteolytic inactivation (Fontes et al., 1995).

Finally, preingestive effects also include the removal of structural barriers that prevent microbial access to fibrous components in the diets. It has been hypothesized that the mode of action of alkaline serine proteases in ruminant diets was related to the removal of structural barriers (Colombatto et al., 2003a). As suggested by Wallace and Kopečný (1983), these barriers could be composed of structural proteins from the cell wall, which might act as barriers to microbial digestion. Colombatto and Beauchemin (2009) suggested that a subtilisin-like protease targeted cross-linkages between structural proteins in alfalfa, and consequently microbial access was increased, resulting in accelerated ruminal fermentation due to the removal of the structural protein.

Supplementing diets with exogenous enzymes is expected to increase the total hydrolytic capacity in the rumen, and it is possible that through this increase, digestion then improves. However, it is very difficult to measure total enzymatic activity in the rumen, which makes this hypothesis difficult to prove. Beauchemin and Rode (1996) calculated that adding exogenous enzymes to feed could potentially increase cellulase activity in the rumen by up to 15%. Also, Wallace et al. (2001) calculated that an enzyme product added to the diet at 1.5 mg/g would increase xylanase activity by 5% and cellulase activity by 15%.

The increase in hydrolytic capacity previously mentioned might be better explained by the activities of both microbial and exogenous enzymes due to potential synergism (Morgavi et al., 2000). This synergy can be defined as the enhanced effect of these two entities acting cooperatively. This means that the increase in enzyme activity exceeds the

additive effects of each of the individual components (microbial and exogenous enzymes). When enzymes from *Trichoderma longibrachiatum* were combined with ruminal enzymes extracted from cattle receiving high fiber or high concentrate diets, hydrolysis of soluble cellulose and xylan increased by up to 35 and 100%, respectively (Morgavi et al., 2000). The hydrolysis of corn silage also increased by 40%. When the individual solubilized monosaccharides were measured, it revealed that exogenous xylanase did not stimulate glucose release, and that exogenous cellulase did not stimulate xylose release when combined with endogenous ruminal enzymes. This result indicates that the synergistic effect was occurring between enzymes that were attacking the same substrate at the same hydrolysis site.

In the literature, numerous potential modes of actions have been suggested for the action of exogenous feed enzymes. However, when seen individually, none of them account for all the increases in diet digestibility reported. It is most likely that these modes of action are interdependent and that a more integrated mode of action that accounts for pre-ingestive effects, increased hydrolytic capacity, and synergistic effects can fully explain increases in diet digestibility when exogenous enzymes are used (Beauchemin et al., 2004).

Beef Cattle Responses to Feed Enzyme Application

Typical growing beef cattle diets have a higher proportion as forage. This forage portion contains 30 to 70% NDF (DM basis), and even under ideal conditions total tract digestibility of NDF is less than 50%. With the addition of exogenous enzymes, fiber digestibility of the forage portion of growing beef diets could be increased, and thus

increasing animal performance at early stages of production. On the other hand, finishing diets contain a higher portion of concentrate, with the main source being grains. Grains contain high amounts of readily fermentable carbohydrates that can lower pH in the rumen to less than 6.0. Optimum pH for fibrolytic bacteria is 6.0 to 6.2, and therefore a pH lower than 6.0 can lower fibrolytic activity in the rumen, thus decreasing fiber digestibility. Some exogenous enzymes employed as feed additives have an optimal pH lower than that of endogenous enzymes. Hence, their addition could be of great benefit when ruminal pH is suboptimal for fibrolytic bacteria (Beauchemin et al., 2004).

The results of adding fibrolytic enzymes to high grain diets have been surprisingly more consistent than those for high forage diets. Applying an enzyme product (Xylanase B, Biovance Technologies Inc., Omaha, NE) to a 95% barley-based diet improved feed efficiency by 6 to 12%, depending upon the level of enzyme addition (Beauchemin et al., 1997, 1999). Increased feed efficiency was due to an increase in diet digestibility (Miller et al., 2008). In addition, Krause et al. (1998) reported a 28% increase in ADF digestibility using a similar enzyme product added to a high concentrate diet. Using another enzyme product (Finnfeeds Int. Ltd., Marlborough, UK), McAllister et al. (1999) reported that treating both the forage and grain portions of the diet with 3.5 L/t of DM increased ADG by 10%. However, ZoBell et al. (2000) reported no effects when what appears to be the same enzyme product was added to a high-grain barley based feedlot finishing diet containing 17% forage (DM basis).

Eun et al. (2009a) determined the growth performance on growing and finishing beef steers when fed with an exogenous fibrolytic enzyme (**EFE**) product (Fibrozyme[®], Alltech Inc., Nicholasville, KY). The supplementation of the EFE product had no effect

on growth performance or carcass characteristics when used at 1 and 2 g/kg DM TMR. Another study done by Ranilla et al. (2008) used the same enzyme product and resulted in a decrease in acetate-to-propionate ratio in grass hay and an increase of the same ratio in alfalfa hay at short in vitro incubation times (5 h). The endoglucanase and xylanase activities reported were greater than those reported by Eun et al. (2009a) which may partially explain the lack of effects on the latter. This highlights the need to measure the enzymatic activities of exogenous enzyme products on all studies.

Digestibilities

Feng et al. (1996) reported increases in total tract DM and NDF digestibilities when applying an enzyme product, consisting mostly of cellulase and xylanase activities, to dry-grass forage right before feeding at a rate of 5.26 mL/kg of DM. This increase in digestibility was associated with an increase in DMI and the combination resulted in a 21% increase in DM digested per day. These results were consistent with in vitro digestibilities reported on the same study (Feng et al., 1996). Overall, application of the enzyme product to dry forage immediately before incubation and before feeding was effective in increasing DM and NDF digestibilities. Greater NDF digestibilities have been found when a commercial enzyme product (Grasszyme[®], FinnFeeds International, Marlborough, Wiltshire, U.K.) was added to a 70% grass hay diet 0 and 24 h pre-feeding at a rate of 1.65 mL/kg of forage DM (Lewis et al., 1996). Digestibility of DM, NDF, and ADF tended ($P = 0.13$) to be greater for steers receiving all enzyme treatments compared with control, and DM, NDF, and ADF digestibilities tended ($P = 0.15$) to be lower when enzyme treatment was ruminally infused rather than applied to the feed at 0 or 24 h prior

to feeding. These studies highlight the fact that one of the mode of actions of enzymes is through pre-ingestive attacks on the forage (Beauchemin et al., 2003).

Miller et al. (2008) found no effects on DM, OM, or fiber digestibilities when an enzyme product was added to the grain portion of high or low concentrate diets at a rate of 4.18 mL/kg diet DM with sorghum as the main grain. In vitro incubations (39°C, pH 6.5, up to 4 h) of the enzyme product used in the study showed that sugar release was greater for barley grain than sorghum grain (Miller et al., 2008). This enzyme/substrate specificity could explain the lack of responses on DM, OM, or fiber digestibilities found on Miller et al. (2008).

Increased ADF digestibility was reported for alfalfa hay at low enzyme dose rates and for timothy hay at higher dose rates (Beauchemin et al., 1995). For the alfalfa hay there were also improvements on DMI and consequently ADG; however, on timothy hay diets, there were no increases on DMI which resulted in an improved feed efficiency. On a study done by Krause et al. (1998) ADF digestibility was improved by 55% for diet containing silage and by 14% for diet containing straw. Part of the improvement in digestibility was attributed to the enzyme improving the digestibility of barley hulls, allowing rumen microbes to access to the highly digestible starch in the barley grain. These studies emphasize strong specificity between enzyme and substrate, which needs to be considered when formulating enzyme products that could potentially work for an array of substrates.

Ruminal Fermentation Characteristics

Lewis et al. (1996) reported an increase on total VFA production at 16 h on steers fed enzyme treated diets. Increases in total VFA were also found at 4 and 12 h when the

enzyme was applied to the barley portion of the diet. The authors attributed these increases to improved ruminal fermentation of structural carbohydrates from barley rather than grass hay.

Total VFA production was reported to be higher with enzyme supplementation at 6 wk, but not at wk 2 when fed to grain diets based on either sorghum grain or dry-rolled barley grain (Miller et al., 2008). The authors also reported that VFA concentration was higher at 4 h post-feeding for enzyme supplementation compared with control. This suggests that with the specific enzyme product, more time is needed in the rumen to elicit positive effects on ruminal fermentation characteristics. Some changes in individual VFA were attributed to N availability and synchrony between energy and N release during a 24 h feeding cycle (Miller et al., 2008).

Enzyme supplementation had no effect on total VFA or molar proportions of VFA when applied to the concentrate portion of the diet (Krause et al., 1998). However, enzyme treatment numerically increased molar proportion of propionate, and tended ($P = .10$) to decrease acetate-to-propionate ratio. These changes in VFA proportions indicate an increase in ruminal starch digestion due to the enzyme addition.

Most studies that analyzed pH parameters reported no significant changes between enzyme treatments and controls (Feng et al., 1996; Krause et al., 1998; Miller et al., 2008). Miller et al. (2008) did report a lower pH when enzyme was applied to a diet containing barley, but the mean pH was still above 6.2. Therefore, fiber digestion was not compromised. However, Lewis et al. (1996) reported that overall pH was lower for enzyme treatment vs. control. Barley based diet supplemented with exogenous enzyme had a ruminal pH of 6.1 for at least 8 h/d, and digestibility of DM, NDF and ADF tended

($P = 0.13$) to be greater for enzyme treatment (Lewis et al., 1996). In this case, enzyme treatment may have minimized depressions in fiber digestion during periods of low ruminal pH by providing activities that would otherwise be lacking. Cellulase-hemicellulase preparations have been found to have an optimal pH for fibrolytic activities as low as 4.5 (Sheperd and Kung, 1994).

In summary, beef cattle responses when exogenous enzymes are added to the diet remain highly variable; however, positive responses on animal performance can be attributed to increased diet digestibility and more specifically increased fiber digestibility due to enzyme supplementation. Total VFA increases with enzyme supplementation, which is expected to increase due to increases in DM and NDF digestibility. However, more in vivo studies are needed to provide a better understanding of the mode of action of enzyme additives when applied to a TMR fed to beef cattle on actual feedlot production settings. These studies will provide more information on the interaction of exogenous enzymes with endogenous microbial enzymes and its effect on passage rate and other in vivo parameters.

In Vitro vs. In Vivo Studies on Feed Enzyme Research

Due to the specificity of enzymes for their substrates, the array of enzyme activities that are supplemented must be very specific to the chemical composition of the targeted forage so that enzymes can then improve forage degradation (White et al., 1993). In addition, exogenous enzymes work synergistically with enzymes from the rumen microbes, which increases their hydrolytic potential within the rumen (Morgavi et al., 2000). So far, it has been extremely difficult to predict the potential of exogenous enzyme

preparations to increase the degradation of cell wall components based only on their biochemical characterization (Colombatto et al., 2003b). Therefore, in vitro systems such as batch culture incubations that allow for measurements of fiber degradability and gas production have been used to identify effective enzyme candidates (Eun and Beauchemin, 2005a). These in vitro assays are less expensive, less time consuming, and allow more control of experimental conditions than in vivo studies. However, they have limitations such as the inability to measure intake that can limit the ability of predicting the true efficacy of an enzyme product.

Eun and Beauchemin (2005a) fed and EPE product (Protex 6L[®]) to dairy cows and found an unexpected decrease in feed intake of cows, which offset the improved feed digestibility. In turn, milk yield of cows fed diets supplemented with the enzyme product decreased. This same enzyme product was used in a batch culture study (Colombatto et al., 2003b) by applying it to alfalfa hay at a similar rate (1.5 mg/g of DM alfalfa hay) as was used in the in vivo study using a TMR (Eun and Beauchemin, 2005a). Degradation of DM at 18 h was improved by 9.8% (Colombatto et al., 2003b). In a continuous culture, NDF degradability was increased by 25 to 43% when the same enzyme was added to a TMR at a rate of 1.5 mg/g of DM TMR (Colombatto et al., 2003a). However, since in vitro studies cannot account for effects of enzyme supplementation on intake, negative responses on intake are unexpected. In the case of cows fed low forage diets, the decrease in feed intake could be due to ruminal acidosis resulting from low ruminal pH. This low ruminal pH could be the result of higher fiber digestibility due to enzyme supplementation.

Future Challenges and Implications on Using Feed Enzymes

One of the main challenges of enzyme technology is to be able to screen different enzyme products before in vivo trials. While in vivo trials are the most effective assessment of whether an enzyme product enhances feed utilization, they come with high costs. In addition, using sheep as a model for cattle is not a viable alternative because of their lower intake and slower rate of passage (Yang et al., 2000). Therefore, there is a need to utilize in vitro bioassays as a way to screen potential candidates for further in vivo trials. In vitro trials will also allow for various candidates and various types of forages to be analyzed on a single study. However, for bioassays to be effective it needs to reflect the conditions of the environment in which the enzyme is expected to function. In this case, pH and temperature that reflect the typical conditions within the rumen should be effective. Also, dose rate of the enzyme, method of application, and processing of the feed should be considered for in vitro bioassays to elicit more responses that could be better extrapolated on to animal trials.

Another challenge is the enzyme-feed specificity when formulating rations, because most commercially used TMR contain various types of forages and concentrates. In this case, an array of enzymatic activities would be needed in order to improve the digestibility of the various ingredients that are included in the TMR. There are two options to this problem. First, a more generalized enzyme product could be added, meaning a product that may not be the best for all forages, but it is relatively suitable for most feeds. However, this generalized approach can elicit variable responses. Therefore, livestock producers will be discouraged from using enzyme products under certain feeding programs, because the enzyme products can cause inconsistent responses in

animal productivity when compared to the high cost of enzyme products. The second approach is a more targeted one in which feed enzyme products are formulated for various types of feed. Although this approach results in a new degree of complexity in the marketplace, it may be the best way to ensure the response of feed enzyme technology on the farm.

There has been a considerable amount of *in vivo* research using feed enzyme products on dairy cattle (Lewis et al., 1999; Rode et al., 1999; Schingoethe et al., 1999; Yang et al., 1999; Beauchemin et al., 2000; Kung et al., 2002; Yang et al., 2000; Bowman et al., 2002; Knowlton et al., 2002; Sutton et al., 2003; Vicini et al., 2003; Eun and Beauchemin, 2005a; Adegosan et al., 2007; Elwakeel et al., 2007; Hristov et al., 2008; Zebeli et al. 2008). However, there has been considerably less research done on feedlot cattle with both growing and finishing periods. With increasing feed prices affecting both dairy and beef industry, enzyme products present an alternative to improve feed efficiency, thereby decreasing the amount of feed necessary for meat production and consequently lowering production costs.

With the expansion of the ethanol industry there is an increase in the production of by-products such as DDGS. This product has become increasingly more available, and it represents a very cost effective feed ingredient for dairy and beef rations. Since the starch component of the corn is removed to produce ethanol, DDGS has a higher portion of NDF but low amounts of lignin. This makes it a readily digestible source of fiber that can serve as a partial replacement for forages as well as concentrates in dairy and beef cattle diets (Schingoethe, 2007) and can supply energy required for lactation or growth without the ruminal acid load cause by rapidly fermented starchy compounds (Ham et al., 1994).

Since DDGS contains high amounts of digestible fiber, the use of exogenous enzymes that could increase the digestibility of fiber could be a possibility to increase fiber digestion of DDGS, allowing it to be better utilized in dairy and beef cattle diets. The addition of cell wall degrading enzymes induces beneficial results on nutrient utilization and growth performance on swine (Pierce and Bannerman, 2008) and poultry (Jackson et al., 2008) when added to DDGS containing diets; however, no studies have been done to assess potential effects of exogenous feed enzymes on ruminant diets containing DDGS. Very recently, Vera et al. (2010) performed a series of batch culture studies using a protease enzyme product (Protex 6L[®]) on DDGS as a pure substrate or beef steer TMR containing 20% DDGS. During a 24 h incubation period, protease addition to DDGS resulted in quadratic responses on degradability of DM, NDF, and ADF, and its optimum dose rate was found to be 1.4 mg/g DM. In a 96 h incubation study, the degradability of NDF and ADF for DDGS increased starting at 18 h of incubation. When the protease was added to beef growing and finishing TMR, NDF degradability of both diets tended ($P = 0.07$) to increase at 12 h of incubation, but the effects were minor at later hours of incubation. This preliminary in vitro study suggests that use of feed enzyme, particularly EPE, may have potential to increase nutritive value of DDGS on ruminant diets and resultant animal performance.

MATERIALS AND METHODS

The animals used in this study were cared for according to the Live Animal Use in Research Guidelines of the Institutional Animal Care and Use Committee at Utah State University.

Enzyme Product

A developmental EPE product from Danisco-Agtech (Waukesha, WI) was used in the current study, and it was in powder form and compliant with current specifications for food-grade enzymes in North America. The product is an alkaline protease enzyme and classified as a serine endopeptidase of the subtilisin family (EC 3.4.21.62).

Exp. 1: Assessment of Growth Performance of Beef Steers in Response to Supplementing EPE during Growing and Finishing Phases

Exp. 1 was undertaken to assess the effects of supplementing an EPE product in growing and finishing diets of beef steers on growth performance and carcass characteristics. The study was conducted in a completely randomized design at the Utah State University animal science research farm (Wellsville, UT) during growing and finishing phases.

Growing phase. Forty-eight Angus crossbred steer calves (initial BW = 257 ± 16.3 kg) were used in this trial. All calves had been processed similarly prior to trial initiation by receiving a Brucellosis vaccination, parasite treatment (Dectomax[®], Pfizer Animal Health, Exton, PA), eight-way Clostridial vaccine (Pfizer Animal Health) and an intranasal respiratory product (BoviShield[™], Pfizer Animal Health). Calves were housed

in groups of 4 in shaded pens (i.e. one treatment per pen), and they received one out of two growing diets: DDGS TMR (**DT**) without EPE (**DT–EPE**) and DT with EPE (**DT+EPE**). This resulted in 6 pens for the treatment and control diets, respectively. The EPE product was diluted with water and added at a rate of 0.52 g/kg DM TMR in order to contain proteolytic activity of 27 mg of azocasein hydrolyzed/min/kg DM TMR, as it was mixing for the DT+EPE treatment. The rate of enzyme application was selected based on our previous in vitro research (Vera et al., 2010). Furthermore, based on the cost of the enzyme, this dose represented an upper threshold at which the product would likely be used commercially as a ruminant feed additive. Applying commercial enzyme products before feeding enhances binding of the enzyme to the feed, preventing proteolysis of the enzyme in the rumen. The diets contained 13.4% alfalfa hay, 50.1% corn silage, 30.3% DDGS, and 6.2% feedlot supplement on DM basis (Table 1). The corn DDGS used in this study was supplied by Cache Commodities (Ogden, UT), and contained $97.0 \pm 0.85\%$ DM, $94.8 \pm 0.21\%$ OM, $28.4 \pm 1.41\%$ CP, $34.3 \pm 0.78\%$ NDF, $11.3 \pm 0.85\%$ ADF, $3.55 \pm 0.212\%$ starch, $12.4 \pm 1.29\%$ fat, $0.20 \pm 0.191\%$ Ca, and $1.05 \pm 0.389\%$ P. Animals were fed to appetite at 0800 h daily.

All steers were allowed to adapt to the DT–EPE diet for a 2-wk period before the beginning of the trial. The steers had free access to fresh water. All feedstuffs were analyzed initially for DM, and DM of corn silage was obtained bi-weekly. The TMR was mixed and delivered to the steers in a feed cart (Rissler Mfg., Mohnton, PA), which recorded the amounts fed daily. All steers were fed once per day (0700 h) to appetite. Feed bunks were read each afternoon and before the morning feeding, and these readings were used to determine the amount of feed to deliver to each pen the following day.

Table 1. Ingredients and chemical composition of beef steer diets used in Exp. 1

Item	Diet	
	Growing	Finishing
Ingredient, %DM		
Alfalfa Hay	13.4	5.0
Corn Silage	50.1	20.0
Barley, dry rolled	-	40.0
Corn DDGS ²	30.3	30.0
Feedlot supplement ¹	6.2	5.0
Nutrient, % DM		
OM	94.8 ± 0.21	94.6 ± 0.153
CP	15.5 ± 0.31	14.8 ± 0.10
NDF	38.3 ± 0.32	30.6 ± 1.62
ADF	22.1 ± 0.10	14.1 ± 0.93
Starch	16.9 ± 0.32	24.5 ± 2.20
Fat	6.68 ± 0.237	5.25 ± 0.263
Ca	0.65 ± 0.068	0.87 ± 0.042
P	0.40 ± 0.064	0.48 ± 0.026

¹Formulated to provide 50 g/kg NaCl, 2.4 g/kg Mg, 7.6 g/kg K, 200 ppm Cu, 400 ppm Mn, 650 ppm Zn, 2 ppm Se, 22 ppm I, 9 ppm Co, 121,000 IU/kg vitamin A, 37,400 IU/kg vitamin D, 55 IU/kg vitamin E, and 360 ppm Rumensin[®] (Elanco Animal Health, Indianapolis, IN).

Feed samples were obtained weekly and composited by month for each treatment.

The DM content of feed was determined by oven drying at 60°C. Mean DMI was determined once per week, and it was calculated for each pen as the total amount of DM allocated daily divided by the number of cattle per pen on that particular day. Thus, intake accounted for any sick cattle removed from the pen during treatment. The assumption was that DMI was the same for all cattle within the pen. All steers were weighed on d 0, 28, 56, and 84. Body weight gain was determined on the same days by comparing the initial and final BW for individual animals, and ADG was calculated during each period. In addition, G:F was calculated as kilograms of ADG divided by kilograms of DMI.

Feed DM and nutrient digestibility was measured on wk 4, 8, and 12 using acid-insoluble ash (**AIA**) as an internal marker (Van Keulen and Young, 1977). Fecal samples (approximately 200 g wet weight) were collected for each animal from the rectum twice daily (a.m. and p.m.) every 12 h, moving ahead 2 h each day for the 6 d of fecal sampling. This schedule provided 12 representative samples of feces for each animal. Samples were immediately subsampled (about 50 g), composited across sampling times for each cow and each period, dried at 55°C for 72 h, ground to pass a 1-mm screen (standard model 4, Arthur Thomas Co., Philadelphia, PA), and stored for chemical analysis. Apparent total tract nutrient digestibilities were calculated from concentrations of AIA and nutrients in diets fed, orts, and feces using the following equation: apparent digestibility = $100 - [100 \times (AIA_d/AIA_f) \times (N_f/N_d)]$, where AIA_d = AIA concentration in the diet actually consumed, AIA_f = AIA concentration in the feces, N_f = concentration of the nutrient in the feces, and N_d = concentration of the nutrient in the diet actually consumed (Eun and Beauchemin, 2005a).

Finishing phase. Growth experiment during the finishing phase consisted of 48 steers (initial BW = 399 ± 26.1 kg) used in the growing phase, and they were fed the same treatments assigned in the growing phase. After finishing the growing phase, the concentrate portions of the diets were gradually increased over a 28-day period in order to contain 5.0% alfalfa hay, 20.0% corn silage, 40.0% dry rolled barley grain, 30.0% DDGS, and 5.0% feedlot supplement (Table 2). All the measurements were conducted in the same manner described in the growing phase, and all the performance data were collected for 84 d. The finishing phase was terminated based on live animal weight and

visual appraisal. The steers were slaughtered at the JBS Swift & Company (Hyrum, UT) facility, and carcasses were graded after a 24 h chill.

Table 2. Ingredients and chemical composition of beef steer finishing diets used in Exp. 2

Item	Diet ¹	
	NDT	DT
Ingredient, % DM		
Alfalfa Hay	5.0	5.0
Corn Silage	20.0	20.0
Barley, dry rolled	60.0	40.0
Corn DDGS ²	-	30.0
Soybean meal	10.0	-
Feedlot supplement ³	5.0	5.0
Nutrient, % DM		
OM	94.5 ± 0.31	94.6 ± 0.153
CP	12.5 ± 0.57	14.8 ± 0.10
NDF	25.1 ± 1.11	30.6 ± 1.62
ADF	13.7 ± 1.65	14.1 ± 0.93
Starch	36.4 ± 2.31	24.5 ± 2.20
Fat	2.24 ± 0.354	5.25 ± 0.263
Ca	0.84 ± 0.156	0.87 ± 0.042
P	0.39 ± 0.032	0.48 ± 0.026

¹NDT = non-dried distillers grains with solubles (DDGS) TMR; and DT = DDGS TMR.

²Formulated to provide 50 g/kg NaCl, 2.4 g/kg Mg, 7.6 g/kg K, 200 ppm Cu, 400 ppm Mn, 650 ppm Zn, 2 ppm Se, 22 ppm I, 9 ppm Co, 121,000 IU/kg vitamin A, 37,400 IU/kg vitamin D, 55 IU/kg vitamin E, and 360 ppm Rumensin[®] (Elanco Animal Health, Indianapolis, IN).

Exp. 2: In Vitro Ruminal Fermentation Characteristics

of Finishing Beef Steer Diets in Response to EPE

Addition in Continuous Cultures

The aim of Exp. 2 was to examine in vitro ruminal fermentation variables to explain some positive responses observed in Exp. 1 and to determine whether efficacy of supplementing EPE would be consistent without or with DDGS inclusion. The design of

the experiment was a 2×2 factorial with 4 independent runs as replicates ($n = 4$), and a fermentor in continuous cultures was considered an experimental unit. Fermentors were randomly assigned to a sequence of 4 diets; non-DDGS TMR (**NDT**) or DT finishing beef steer diet with a forage-to-concentrate ratio of 25:75 (DM basis), was combined without or with EPE to form 4 treatments: NDT without EPE, NDT with EPE, DT without EPE, and DT with EPE (Table 2). The DT was the same diet used as a finishing diet in Exp. 1. Before use in the fermentors, the diets were dried at 55°C for 48 h and ground through a 4.0 mm screen (standard model 4). For application of the enzyme, exactly 0.5 g of each enzyme powder was solubilized using 50 mL of water, and 520 μ L of the diluted enzyme was added to 10 g (DM basis) TMR (stored in 250-mL plastic containers) using a pipette. The control treatments received 520 μ L of distilled water. Upon enzyme addition, the TMR in the plastic containers was mixed by inversion several times. Enzyme-feed interaction time ranged between 12 and 24 h at 4°C. The dose rate of the EPE was exactly same as the one used in Exp. 1.

A single run was composed of 4 fermentors that were inoculated simultaneously with ruminal contents obtained from two ruminally fistulated, dry cows fed a forage diet. Ruminal fluid was collected 4 h after the morning feeding (1100 h). Grab samples of ruminal contents were obtained from various locations within the rumen and composited. The ruminal contents were placed in sealed, preheated containers and transported to the lab, where the contents were strained through polyester screen (PeCAP, pore size 355 μ m; B & SH Thompson, Ville Mont-Royal, QC). Each of 4 fermentors received approximately 700 mL of strained ruminal fluid under a stream of oxygen-free CO₂. A dual flow continuous culture system based on Teather and Sauer (1988) was used, and it

consisted of 1-L gas-tight fermentor vessels (Prism Research Glass, Inc., Research Triangle Park, NC). A constant flow of CO₂, delivered at 20 mL/min, maintained anaerobic fermentation conditions. Over a 24-h period, artificial saliva (Slyter et al., 1966) was delivered to each fermentor to yield a fractional dilution rate of 8.0%/h (1.2 mL/min) by precision pump (Model 323, Watson-Marlow Inc., Wilmington, MA). The temperature of the cultures was maintained at 39°C by a circulating water bath.

Each independent run lasted 10 d (8 d of treatment adaptation and 2 d of data and sample collection). The first 3 d of each run allowed for microbial adaptation to the diet, with experimental diets gradually replacing alfalfa hay. From d 5, all fermentors received a full experimental diet. Therefore, all fermentors had an adaptation period with full experimental treatments (assigned dietary treatment) for 4 d. Each fermentor received 20 g/d (DM basis) of the corresponding experimental diet in 2 equal portions being added to each fermentor at 0800 and 2000 h. Diets were manually fed to the fermentor through a feed port on the fermentor vessel. Data and samples were taken on d 9 to 10.

All data collection, sampling, and analysis were independently performed in each run. Culture pH was recorded through a pH electrode connected to a pH meter (model 63, Jenco Instruments, Inc., San Diego, CA) every hour for 12 h on d 9 and 10. Methane (CH₄) samples were taken from the headspace gas of each fermentor at 0, 3, 6, 9, and 12 h after the morning feeding using a 10 µL gastight syringe (Hamilton Co., Reno, NV) and analyzed for CH₄ with a GLC (model CP-3900, Varian, Walnut Creek, CA). Daily CH₄ output (mmol/d) was calculated as reported earlier (Williams et al., 2010) using the following equation: CH₄ concentration in fermentor headspace (mmol/mL) × CO₂ gas flow through the fermentor headspace (20 mL/min) × 60 min × 24 h. Immediately after

CH₄ sampling, 5 mL of fermentor ruminal fluid was collected, filtered, added to 1 mL of 1% sulfuric acid, and retained for ammonia-N ($\text{NH}_3\text{-N}$) determination. Another 5 mL of ruminal fluid taken at 3, 6, and 9 h was added to 1 mL of 25% meta-phosphoric acid, and samples were retained for VFA determination. These samples were stored at -40°C until analyses. Overflow from each fermentor was collected in a sealed bottle that was kept on ice to prevent fermentation, and collected every 24 h on d 9 and 10 to determine apparent digestibility of the diets. The overflow culture content was strained through polyester screen (i.e., PeCAP, pore size 355 μm), and only the particulate fraction was retained and analyzed for DM, OM, and NDF.

Chemical Analyses

Analytical DM concentration of samples was determined by oven drying at 135°C for 3 h; OM was determined by ashing, and N content was determined using an elemental analyzer (LECO TruSpec N, St. Joseph, MI) (AOAC, 2000). The NDF and ADF concentrations were sequentially determined using an ANKOM^{200/220} Fiber Analyzer (ANKOM Technology, Macedon, NY) according to the methodology supplied by the company, which is based on the methods described by Van Soest et al. (1991). Sodium sulfite was used in the procedure for NDF determination and pre-treatment with heat stable amylase (Type XI-A from *Bacillus subtilis*; Sigma-Aldrich Corporation, St. Louis, MO). Starch content of diets was determined by a two-step enzymatic method (Rode et al., 1999) with a microtiter plate reader (MRX[°], Dynex Technologies, Chantilly, VA) to read glucose release colorimetrically at 490 nm. Calcium and phosphorus of the feed samples were analyzed using methods described by Isaac and Johnson (1985).

The amount of protein present in the enzyme products was determined using the Bio-

Rad DC protein determination kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as the standard according to Colombatto et al. (2003b). The enzyme products were analyzed for their endoglucanase (EC 3.2.1.4) and xylanase (EC 3.2.1.8) activity according to procedures reported by Wood and Bhat (1988) and Bailey et al. (1992) using medium-viscosity carboxymethylcellulose and birchwood xylan (10 mg/mL in 0.1 mol citrate phosphate buffer, pH 6.0), respectively, as a substrate. Assay conditions were 39°C and pH 6.0 to reflect ruminal conditions. Protease activity was assayed using azocasein (lot 25H7125, Sigma-Aldrich Corporation) in 0.1 mol citrate phosphate buffer (pH 6.8) as a substrate in a similar manner as used by Brock et al. (1982) and Eun and Beauchemin (2005a). Protease activity was expressed as mg of azocasein hydrolyzed/min.

Concentration of $\text{NH}_3\text{-N}$ was determined as described by Rhine et al. (1998) using a microplate reader. Ruminal VFA were separated and quantified using a GLC (model 6890 series II; Hewlett Packard Co., Avandale, PA) with a capillary column (30 m \times 0.32 mm i.d., 1 μm phase thickness, Zebron ZB-FAAP, Phenomenex, Torrance, CA) and flame-ionization detection. The oven temperature was held at 170°C for 4 min, increased to 185°C at a rate of 5°C/min, then increased by 3°C/min to 220°C and held at this temperature for 1 min. The injector and the detector temperatures were 225 and 250°C, respectively, and the carrier gas was helium (Eun and Beauchemin, 2007).

Statistical Analyses

Data for this study was analyzed using the Proc Mixed procedure of SAS (SAS Institute, 2007). In Exp. 1, pen was the experimental unit with monthly data collection periods as repeated measures of treatments. Data for ADG, DMI, and G:F were analyzed

using the following model: $Y_{ijk} = \mu + T_i + P_j(T)_i + M_k + TM_{ik} + \varepsilon_{ijk}$ where, μ = overall mean, T_i = fixed effect of dietary treatment i, $P_j(T)_i$ = random effect of pen j within dietary treatment i, M_k = effect of sampling month k, TM_{ik} = interaction between dietary treatment i and sampling month k, and ε_{ijk} = residual error. Because interactions were lacking in all cases, data were reanalyzed using a model that included treatment as a fixed effect and the random effect of pen, with months as repeated measures of the treatments. Simple, autoregressive one, and compound symmetry covariance structures were used in the analysis depending on low values for the Akaike's information criteria and Schwartz's Bayesian criterion. Data for BW and carcass characteristics were analyzed, with the random variable being the pen within treatment using the following model: $Y_{ij} = \mu + T_i + P_j(T)_i + \varepsilon_{ij}$, where Y_{ij} = individual response variable measured, μ = overall mean, T_i = fixed effect of dietary treatment i, $P_j(T)_i$ = random effect of pen j within dietary treatment i, and ε_{ij} = residual error.

In Exp. 2, data for VFA and digestibility were analyzed using the following model: $Y_{ijkl} = \mu + R_i(F_j) + T_k + E_l + (TE)_{kl} + e_{ijkl}$, where Y_{ij} = individual response variable measured, μ = overall mean, $R_i(F_j)$ = random effect of fermentor j within independent run i, T_k = fixed effect of TMR k (NDT vs. DT; k = 1 to 2), E_l = fixed effect of enzyme l (without vs. with EPE; l = 1 to 2), $(TE)_{kl}$ = interaction between TMR k and enzyme l, and e_{ijkl} = residual error. Denominator degrees of freedom were estimated using the Kenward-Roger option. The same mixed model was used for variables that were repeated in time (culture pH and CH₄), but sampling time and a repeated statement were added to the model. One of 3 model structures was used depending on the finite-sample corrected Akaike's information criterion value for data that best fit the model. The structures were

unstructured and compound symmetry, unstructured and first-order autoregressive, and unstructured and unstructured variance-covariance structure.

Least squares means were generated and separated using the PDIFF option of SAS for the main effect. Significant effects of the treatment were declared if $P < 0.05$, and trends were accepted if $0.05 < P < 0.15$.

RESULTS AND DISCUSSION

EPE Product

The EPE product used in the current study was produced by a strain of *B. subtilis*. It had broad specificity and hydrolyzed peptide amides (Aehle, 2004). In a previous in vitro study (Eun et al., 2007), the same EPE product (formerly denoted as P1) increased gas production by 5.6–7.9% during 24 h of in vitro incubation with alfalfa hay, and NDF degradability increased by 11% at 18 h of incubation. Protein concentration of the EPE product was 87 mg/g. There was little endoglucanase activity (3.0 nmol of glucose released/min/mg), and we did not detect any xylanase activity on the EPE product. The EPE product contained the activity level of 27 mg of azocasein hydrolyzed/min/mg of enzyme product. Thus, the EPE product contained mainly proteolytic activity, but negligible fibrolytic activities.

Recently, we performed a series of in vitro batch culture experiments to assess if an EPE product (Protex 6L, Genencor Division of Danisco, Rochester, NY) would improve degradation of DDGS and growing and finishing beef steer TMR containing 20% DDGS on a DM basis (Vera et al., 2010). The EPE addition in DDGS resulted in quadratic responses on degradability of DM, NDF, and ADF, and its optimum dose rate was found at 1.4 mg/g DM. When the EPE was added in growing and finishing TMR, the EPE addition tended to increase ($P = 0.07$) NDF degradability of growing and finishing TMR at 12 h of incubation, but the effect of EPE on fiber degradation of beef diets was minor at the later hours of incubation. Thus, the improvements in in vivo and in vitro DM and NDF digestibility observed in the present study are consistent with those observed in previous in vitro experiments. These results highlight importance of assessing the

efficacy of exogenous feed enzymes on feed digestion using in vitro techniques prior to conducting in vivo experiments.

Exp. 1

The addition of EPE during the growing phase increased DMI, but had no effects on final BW, BW change, ADG, and G:F (Table 3). Adding EPE during the growing phase decreased NDF digestibility, whereas the digestibility of DM, CP, and ADF was not affected.

Table 3. Growth performance and total tract digestibility of beef steers fed dried distillers grains with solubles (DDGS)-containing diet without or with exogenous proteolytic enzyme (EPE) supplementation in growing phase (Exp. 1, n = 6)

Item	Diet ¹		SEM	P
	DT-EPE	DT+EPE		
BW				
Initial, kg	292	292	2.0	0.89
Final, kg	434	440	6.0	0.39
Change, kg	142	147	5.4	0.37
ADG	1.65	1.67	0.09	0.85
DMI	11.5	13.2	0.63	0.02
G:F	0.143	0.127	0.013	0.66
Digestibility, %				
DM	72.4	72.0	0.69	0.64
CP	71.1	69.6	0.94	0.16
NDF	59.3	56.9	0.78	0.05
ADF	52.1	49.3	1.43	0.19

¹DT-EPE = DDGS diet without EPE and DT+EPE = DDGS diet with EPE.

In finishing phase, final BW ($P = 0.11$) and ADG ($P = 0.09$) tended to increase due to EPE addition (Table 4), but BW change and G:F were not influenced by EPE addition. Furthermore, digestibility of DM, N, NDF, and ADF increased due to EPE addition. The increases in ADG could be due to the higher DM and nutrient digestibility; higher digestibility provides more nutrients for the animals and can increase ADG.

Table 4. Growth performance and total tract digestibility of beef steers fed dried distillers grains with solubles (DDGS)-containing diet without or with exogenous proteolytic enzyme (EPE) supplementation in finishing phase (Exp. 1, n = 6)

Item ²	Diet ¹		SEM	<i>P</i>
	DT-EPE	DT+EPE		
BW				
Initial, kg	470	477	5.1	0.38
Final, kg	593	607	5.8	0.11
Change, kg	123	131	5.8	0.37
ADG	1.75	1.96	0.09	0.09
DMI	12.8	13.3	0.24	0.13
G:F	0.141	0.148	0.010	0.63
Digestibility, %				
DM	53.8	61.1	1.84	< 0.01
N	56.2	62.6	1.85	< 0.01
NDF	39.7	44.4	2.13	0.03
ADF	31.5	36.0	1.87	0.01

¹DT-EPE = DDGS diet without EPE and DT+EPE = DDGS diet with EPE.

Increased total tract N digestibility due to EPE supplementation in finishing diet is worthy of discussion, as it may contribute to improving utilization of dietary N and decreasing N excretion into feces. Eun and Beauchemin (2005a) reported an increase in total tract N digestibility as a result of adding EPE to dairy diets due to increased ruminal degradability of feed N. However, the increased N digestibility did not affect efficiency of feed N use to milk protein. The authors speculated that no improvement on the N use efficiency may have resulted from reduced contribution of RUP to the metabolizable protein pool due to enhanced degradation of CP in the rumen in response to EPE addition (Eun and Beauchemin, 2005a). Hence, increase N digestibility found in this study may have limited impacts on BW gain. On the other hand, the increased N digestibility supports one possible mode of action toward protease proposed by Colombatto et al. (2003a,b) who suggested that protease may remove some of the cell wall N containing

components that act as physical barriers to fiber degradation. The increase in N digestibility in the current study could indicate the degradation of this cell wall N containing components by the action of EPE which then give ruminal microbes more access to the fiber components of the TMR.

A critical factor concerning the efficacy of EPE in ruminant diets is that effects of protease depend on type of target forage like fibrolytic enzymes. Eun and Beauchemin (2005a) demonstrated that although protease improved in vitro degradation of alfalfa hay and barley silage, protease was more effective for alfalfa hay than barley silage. Different responses to protease among forages were reported previously by Colombatto et al. (2003b) who observed that the same protease product was effective when used with alfalfa hay, but not with corn silage. McGinn et al. (2004) reported no effect of this product on total tract digestibility or intake when fed to beef cattle receiving a diet containing 75% barley silage (DM basis). Furthermore, Eun and Beauchemin (2005b) reported that adding protease improved in vitro degradation of alfalfa hay, but not alfalfa silage. In a lactation dairy study, Eun and Beauchemin (2005a) reported that addition of the same EPE product to a low forage diet (18.2% barley silage, 16.0% alfalfa hay, and 65.8% concentrate on DM basis) increased total tract NDF digestibility by 26%, but there was no effect on NDF digestibility when the EPE was added to a high forage diet (44.5% barley silage, 16.0% alfalfa hay, and 39.5% concentrate on DM basis). The cause of the lower or non-efficacy of protease to ensiling forages is not clear, but it may be due to higher quality of silages or fermentation acids produced during the ensiling process. Thus, no effects of adding EPE to the growing diet tested in this study are likely to be related to dietary proportion of corn silage (50 and 20% in growing and finishing diet,

respectively); the higher proportion of corn silage in the growing diet may dilute potential effects of EPE on nutrient digestion.

Addition of EPE did not affect carcass characteristics except that EPE addition tended to increase ribeye area ($P = 0.09$; Table 5). Research on the effects of adding feed enzymes on carcass characteristics has been limited. Commercial enzyme preparations affect diet digestibility and growth rate of cattle and, therefore, only indirect effects on carcass quality are expected (Beauchemin et al., 1997). These results were also expected in this study, implying that EPE supplementation can be used to improve digestibility and animal performance while still maintaining carcass quality of beef steers. Eun et al. (2009a) reported that fibrolytic enzyme supplementation decreased 12th-rib fat thickness and reduce marbling score due possibly to changes in ruminal fermentation mediated by the fibrolytic enzyme. It remains to be determined how the changes in ruminal fermentation by supplementing feed enzymes influence carcass composition in finishing beef steers.

Table 5. Carcass characteristics of beef steers fed dried distillers grains with solubles (DDGS)-containing diet without or with exogenous proteolytic enzyme (EPE) supplementation (Exp. 1, n = 6)

Item	Diet ¹		SEM	<i>P</i>
	DT-EPE	DT+EPE		
Yield grade, %	2.65	2.61	0.146	0.84
HCW, kg	369	373	2.89	0.33
Marbling score ²	501	480	19.6	0.45
Ribeye area, cm ²	76.4	80.6	1.59	0.09

¹DT-EPE = DDGS diet without EPE; DT+EPE = DDGS diet with EPE.

²Practically devoid = 100 to 199, slight = 200 to 299, small = 300 to 399, modest = 400 to 499, moderate = 500 to 599.

Exp. 2

Overall culture pH averaged 5.9 across the treatments (Figure 1). Wilson et al. (2008) reported similar culture pH (6.0) when a typical finishing beef steer diet was incubated in continuous cultures. The culture pH varied following feed provision, but the pattern of diurnal fluctuation of the culture pH was similar among treatments, with the highest pH values observed just before feed input and the lowest pH values 4 h after provision. The pH curves were relatively flat for the NDT compared with the DT diet, and feeding the DT diet decreased culture pH compared with the NDT diet (5.8 vs. 6.0) regardless of EPE supplementation. Similarly, Li et al. (2011) reported that increased replacement of barley silage and barley grain with wheat DDGS linearly decreased mean ruminal pH. The decreased ruminal pH by feeding the DT diet contrasts to our expectation that substitution of a nonstarch concentrate (corn DDGS) for a source of highly fermentable starch (barley grain) should increase ruminal pH. The corn DDGS used in this study seemed to be readily available for ruminal fermentation, resulting in decreased culture pH. Supplementation of EPE did not affect culture pH. Colombatto et al. (2003a) also reported that EPE addition did not affect ruminal pH at any time under the conditions of highly or lowly controlled culture pH. Total VFA concentration was higher for the DT than for the NDT diets (Table 6). This corresponds to pH data, indicating that increased total VFA concentration in the ruminal fluid resulted in decreased culture pH. Zhang et al. (2010) reported no differences in total VFA concentration when DDGS partially replaced barley silage or barley grain in lactation dairy diets. Eun et al. (2009b) reported a tendency ($P = 0.09$) for total VFA to decrease when DDGS partially replaced barley grain in beef steer diets. In contrast, Li et al. (2011) observed that feeding finishing

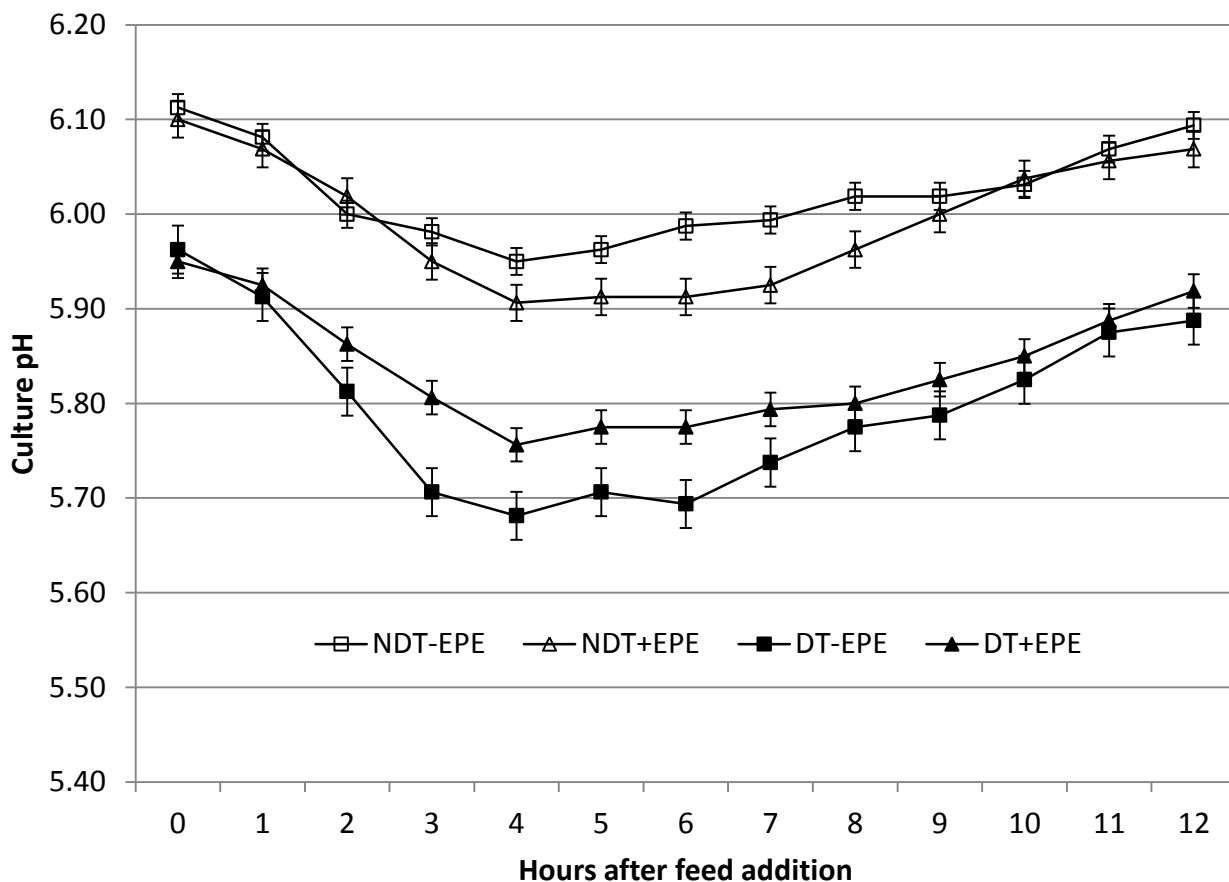


Figure 1. Diurnal fluctuation of pH in continuous cultures receiving finishing beef steer diets without or with exogenous proteolytic enzyme (EPE) supplementation (Exp. 2, $n = 4$). NDT-EPE = non-dried distillers grains with solubles (DDGS) TMR without EPE; NDT+EPE = non-DDGS TMR with EPE; DT-EPE = DDGS TMR without EPE; and DT+EPE = DDGS TMR with EPE. Least square mean for culture pH was 6.02, 5.99, 5.80, and 5.84 for NDT-EPE, NDT+EPE, DT-EPE, and DT+EPE, respectively. Effects of TMR, enzyme, and interaction between TMR and enzyme were $P < 0.01$, $P = 0.31$, and $P = 0.51$, respectively. The SEM were 0.180, 0.202, 0.180, and 0.134 for NDT-EPE, NDT+EPE, DT-EPE, and DT+EPE, respectively.

beef steer diet containing 30% DDGS increased total VFA concentration compared with the diet without DDGS (135 vs. 149 mM). The increase in total VFA concentration found in our study could be due to a higher digestibility of nutrients, allowing more substrate to be fermented by ruminal microbes, thereby increasing total VFA concentration.

Total VFA concentration tended ($P = 0.07$) to increase with EPE supplementation regardless of TMR. Likewise, Eun and Beauchemin (2005a) reported increased total VFA concentration when lactating dairy cows were fed a low forage diet supplemented with an EPE. Giraldo et al. (2007) found an increase of VFA production when fibrolytic enzyme preparations were added in high forage diets. Also, Miller et al. (2008) observed increased VFA production at 4 h post feeding when a mixed xylanase and endoglucanase enzyme product was supplemented in grain-based diets. The increase in VFA production indicates an increased diet digestibility when EPE was supplemented, which in turn could be due to an increase in total hydrolytic capacity in ruminal fermentation due to EPE supplementation. Eun and Beauchemin (2005a) reported that supplementing EPE increased endoglucanase, xylanase, and protease activities in ruminal fluid from cows fed a low forage diet, resulting in increased VFA concentration.

Proportion of acetate tended to decrease ($P = 0.07$) when the DT was fed, whereas proportion of propionate was not affected by diet (Table 6). Acetate-to-propionate ratio was not affected by diet. While feeding the DT diet tended to decrease ($P = 0.10$) butyrate proportion, valerate and isovalerate proportions increased by feeding the DT compared with the NDT diet. Leupp et al. (2009a) reported a linear decrease in acetate proportion when feeding increasing levels of DDGS on a 70% concentrate diet. Vander Pol et al. (2009) also reported decreased acetate proportion in steers fed 40% wet distillers grains with solubles compared with those fed a composite of corn bran and corn gluten meal or corn oil (95% concentrate diets). It is likely that impacts of feeding DDGS on VFA profiles depend on level of DDGS inclusion and composition of other ingredients in the diet. Addition of EPE decreased acetate proportion regardless of diet

(Table 6), while EPE addition did not affect propionate proportion, leading to decreased acetate-to-propionate ratio. It is not uncommon to observe changes in VFA proportions as a direct effect of added enzyme preparation, which could affect the microbial growth and/or shift the metabolic pathways by which specific microbes utilize substrates (Eun and Beauchemin, 2007).

Feeding the DT diet increased DM, OM, and NDF digestibility compared with the NDT. In general, the increased digestibility corresponds to VFA and pH data; higher digestibility resulted in more VFA production, and thus lower culture pH for the DT diet. Leupp et al. (2009a) reported decreased ruminal NDF digestibility when DDGS was replaced for dry rolled corn grain in finishing beef steer diet at 30% DM due to increased NDF intake. Eun et al. (2009b) reported that NDF digestibility tended ($P = 0.14$) to increase with feeding finishing beef steer diet containing 30% DDGS compared with a diet without DDGS. Likewise, Li et al. (2011) observed that NDF digestibility was greater in steers fed the 30% wheat DDGS than those fed a control diet. The greater NDF digestibility of the DT compared with the NDT diet may be explained by the large fraction of digestible NDF in corn DDGS (Ham et al., 1994; Vander Pol et al., 2009), a property that reflects the reduced lignin content of corn DDGS. Nuez-Ortin and Yu (2010) reported greater in situ 48-h disappearance of NDF from corn DDGS (79%) vs. corn (45%) and from wheat DDGS (64%) vs. wheat (51%). Li et al. (2011) observed that in situ ruminal NDF disappearance of wheat DDGS did not support the notion of greater ruminal NDF digestion in steers fed 30% DDGS. However, sizable increase of NDF digestibility by 13% observed in this current study due to 30% DDGS inclusion clearly indicate enhanced ruminal NDF digestion.

Supplementing EPE increased digestibility of DM, OM, and NDF when added to the DT, but not the NDT diet, leading to tendencies on TMR \times enzyme interaction ($P < 0.10$). The positive effects on the digestibilities with the DT diet correspond to those observed in Exp. 1. However, it is difficult to explain the increased digestibility only with the DT diet. 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 (Colombatto and Beauchemin, 2009). 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). For the case of feeding the DT diet, reduced lignin content of corn DDGS may provide relatively easy access for EPE to degrade its target substances.

Production of CH₄ tended to increase ($P = 0.10$) for the DT when compared to the NDT diet. Supplementation of EPE decreased CH₄ production when added in the NDT, but not in the DT diet, resulting in an interaction between TMR and enzyme. Therefore, decreased CH₄ production by feeding the NDT diet was a result of EPE impact on the NDT diet. McGinn et al. (2009) reported that the addition of DDGS (35% DM) in growing beef steer diet reduced CH₄ emissions by 16.4%. The authors attribute the lower CH₄ emission of cattle fed DDGS to the high lipid content of DDGS (12.7% DM), which, when added to the diet, increased the crude fat concentration from 2.0 to 5.1% DM. Likewise, the DT had higher crude fat concentration than the NDT diet due to 30% inclusion of DDGS (12.3% crude fat) in our study. Therefore, it is possible that EPE addition to DDGS-containing diets may prevent potential effect of EPE from decreasing CH₄ production due to a possible interaction between EPE and fat from DDGS

Table 6. Ruminal fermentation characteristics in continuous cultures receiving beef steer finishing diets without or with exogenous proteolytic enzyme (EPE) supplementation (Exp. 2, n = 4)

Item ¹	Diet ²				SEM	Significance of effect ³		
	NDT		DT			T	E	T × E
	-EPE	+EPE	-EPE	+EPE				
Total VFA, mM	50.2	56.1	58.9	61.7	3.65	0.01	0.07	0.48
Individual VFA								
Acetate (A)	41.4	39.4	39.8	38.2	0.73	0.07	0.02	0.82
Propionate (P)	43.2	43.2	42.2	44.5	0.79	0.84	0.16	0.17
Butyrate (B)	10.1	11.0	10.0	9.4	0.57	0.10	0.74	0.16
Valerate	4.09	5.38	6.50	6.16	0.542	< 0.01	0.34	0.11
Isobutyrate	0.49	0.48	0.44	0.50	0.064	0.64	0.46	0.37
Isovalerate	0.45	0.30	0.80	0.95	0.121	< 0.01	0.99	0.14
A:P	0.96	0.92	0.95	0.87	0.029	0.24	0.04	0.49
Digestibility, %								
DM	74.0	73.2	76.2 ^b	80.2 ^a	1.95	0.01	0.17	0.07
OM	72.2	71.5	74.9 ^b	78.0 ^a	2.07	0.01	0.19	0.09
NDF	52.2	53.9	58.4 ^b	61.3 ^a	1.17	0.01	0.10	0.09
CH ₄ , mmol/d	3.86 ^a	2.49 ^b	3.70	3.92	0.392	0.10	0.13	0.04
NH ₃ -N, mg/100 mL	4.60	4.82	4.42	4.92	0.393	0.26	0.55	0.83

^{a,b}Means in the same row within NDT and DT subgroups with different superscripts differ based on single degree of freedom contrasts ($P < 0.05$).

¹Individual VFA expressed as mol/100 mol. CH₄ = methane. NH₃-N = ammonia-N.

²NDT-EPE = non-dried distillers grains with solubles (DDGS) TMR without EPE; NDT+EPE = non-DDGS TMR with EPE; DT-EPE = DDGS TMR without EPE; and DT+EPE = DDGS TMR with EPE.

³T = effect of TMR (NDT vs. DT), E = effect of enzyme (without vs. with EPE), and T × E = interaction between T and E.

on ruminal fermentation and methanogenesis.

The focus to date of feed enzyme technology has been on developing enzyme additives that improve fiber digestion and productive performance, but it may also be possible to develop enzyme additives that reduce CH₄ emissions. Increased fiber

digestion in the rumen typically increase CH₄ production due to increased acetate-to-propionate ratio, whereas some enzymes that improve fiber digestion decreased the acetate-to-propionate ratio in ruminal fermentation (Eun and Beauchemin, 2007), which is thought to be the primary mechanism whereby enzymes decrease CH₄ production. In addition, increased availability of simple sugars due to exogenous feed enzyme treatment on forages can be utilized by the animal and/or ruminal lactate- and propionate-producing bacteria. Increasing the competitiveness of these bacteria against acetate producers can reduce ruminal CH₄ production (Dong et al., 1999). Colombatto et al. (2003a) reported remarkable increases in NDF degradability (43% and 26% at high and low pH, respectively) using a EPE product without increasing the CH₄ production, suggesting that feed enzyme products have significant potential to be included as a feed additive for ruminants without affecting CH₄ production. In contrast, Dong et al. (1999) found an increase in in vitro CH₄ production as a result of addition of a fibrolytic enzyme to orchardgrass hay, although the enzyme preparation increased digestibilities of OM, cellulose, and hemicelluloses by 9, 15, and 20%, respectively. The potential effect of feed enzymes as a means of mitigating enteric CH₄ emissions may depend on mode of action on a specific feed enzyme product interested. More research is needed to characterize more fully the interactive relationship between methanogenic bacteria and fermentative microorganisms under ruminal conditions when EPE products are supplemented in beef cattle diets.

Concentration of ammonia-N was not affected by diet and EPE (Table 6). With the consideration of the fact that a primary target of EPE would be dietary N, no effect of EPE on NH₃-N concentration is somewhat surprising. However, it is possible that the

increased digestibility due to EPE supplementation may increase microbial populations which require more ammonia as a source of N, leading to no apparent difference on ruminal $\text{NH}_3\text{-N}$ concentration compared with no EPE treatment.

IMPLICATIONS

Because addition of the EPE product assessed in this study resulted in some positive responses on in vivo and in vitro experiments when added to finishing beef steer diets, it is clear that use of protease enzyme products may be more effective in high concentrate diets such as finishing beef steer diets. Although addition of EPE improved in vitro and in vivo NDF digestibility in DDGS-containing TMR, caution should be exercised to elicit consistent efficacy of feed protease enzymes due to large variation on nutritive quality of DDGS. There have been reports indicating large variations of fiber, CP, and other feed components from large number of corn DDGS samples due possibly to large differences in the process of manufacture between ethanol plants. This could provide some difficulty when tailoring specific EPE products for DDGS-containing diets and could result in performance and digestibility variations.

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APPENDIX

September 20, 2011

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Dr. Alexandra H. Smith
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Dr. Smith,

I am preparing my thesis in the Animal, Dairy and Veterinary Sciences Department at Utah State University. I hope to complete my degree in September 2011.


I am requesting your permission to include the paper titled: Effects of an exogenous proteolytic enzyme on growth performance of beef steers and *in vitro* ruminal fermentation in continuous cultures, of which you are a coauthor.

Please indicate your approval of this request by signing in the space provided, attaching any other form or instruction necessary to confirm permission. If you have any questions, please call me at the number above. Thank you.

Sincerely,

Juan M. Vera

I hereby give permission to Juan M. Vera to reprint the requested article in his thesis.

Name: Alexandra H Smith Signature:  _____

Date: 28 September 2011 _____