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Effect of adding cofactors to exogenous fibrolytic enzymes on preingestive hydrolysis, in vitro digestibility, and fermentation of bermudagrass haylage

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ABSTRACT

Our objectives were to examine if adding metal ion cofactors (COF) to exogenous fibrolytic enzymes (EFE) would increase the beneficial effects of the EFE on the preingestive hydrolysis and in vitro digestibility and fermentation of bermudagrass haylage. In experiment 1, 5 COF (Mn^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, and Mg^{2+}) were screened to select the best candidates for synergistically enhancing release of water-soluble carbohydrates (WSC) from bermudagrass haylage by 5 EFE. The 5 EFE (1A, 2A, 11C, 13D, and 15D) were sourced from *Trichoderma reesei* and *Aspergillus oryzae* and they were the most effective of 12 EFE at increasing the neutral detergent fiber digestibility of bermudagrass haylage in a previous trial. Adding 1 m*M* of each of the COF to EFE 2A or 11C synergistically increased release of WSC from bermudagrass haylage, as did adding (1 m*M*) Fe^{2+} to 1A, Mn^{2+}, Co^{2+}, or Fe^{2+} to 13D, or Co^{2+}or Fe^{2+} to 15D. The greatest release of WSC responses were obtained by adding Mn^{2+} to 11C (38%) or by adding Fe^{2+} to 2A or 13D (10 and 21.9%, respectively). In experiment 2, the effect of increasing the COF dose on in vitro digestibility and fermentation of bermudagrass haylage was examined using the best EFE-COF combinations from experiment 1. Effects of adding increasing doses of these COF on EFE-mediated changes in vitro digestibility depended on the COF-EFE combination. Adding 10 m*M* Mn^{2+} alone to bermudagrass haylage increased DMD and NDFD by 2.7 and 6.3% and adding 11C alone increased these measures by 6.6 and 15.5%, respectively. However, adding 10 m*M* Mn^{2+} with 11C resulted in 3.5 and 8.1% increases in DMD and NDFD, respectively, beyond the increases caused by adding 11C alone. Adding Fe^{2+} to 2A had no effects on EFE-mediated digestibility responses, but 2A prevented adverse effects of adding Fe^{2+} alone on DMD and NDFD. In contrast, adding Fe^{2+} to 13D reduced the increases in DMD and NDFD caused by adding the EFE alone. This study shows that adding COF to EFE can synergistically increase, decrease, or not affect the hydrolytic effects of EFE on bermudagrass haylage cell walls. The outcome depends on the specific EFE-COF combination and the COF dose. More research is required to understand the mechanisms resulting in these outcomes to exploit beneficial effects of COF on EFE.

Key words: fibrolytic enzyme, dairy cattle, bermudagrass, cofactor

INTRODUCTION

Exogenous fibrolytic enzymes (**EFE**) have been applied to dairy cattle diets to improve animal performance because of their ability to catalyze the depolymerization of forage fiber, which is the main barrier to nutrient availability (Meale et al., 2014). However, the results of EFE application in the literature have been equivocal (Beauchemin and Holtshausen, 2010; Adesogan et al., 2014). This is because the outcome is influenced by numerous factors including the EFE dose (Eun et al., 2007) and activity composition (Eun and Beauchemin, 2007), the prevailing pH and temperature (Arriola et al., 2011), the animal performance level (Schingoethe et al., 1999), the experimental design (Adesogan et al., 2014), and the fraction and proportion of the diet to which the EFE is applied (Krueger et al., 2008; Dean et al., 2013). Despite their well-known effects on the activity of certain enzymes (Voet et al., 2010), effects of metal ion cofactor (**COF**) addition to EFE on the digestibility of forages are unknown. Metal-activated enzymes and metalloenzymes use COF for structural and catalytical roles, respectively (Voet et al., 2010). Metal-activated enzymes require COF for maximal activity because they improve the conformational stability of the enzymes, but the COF are not direct components of the enzyme reaction mechanism (Glusker, 2011). Consequently, metal-activated enzymes can function without COF at a reduced activity level (Glusker, 2011). Whereas metalloenzymes require COF at their active sites to function because they serve as substrate templates, inducers of free radicals, and

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redox-active COF (Purich, 2011). Among enzymes involved in lignocellulose degradation, only a few, mostly those grouped in the auxiliary activities family, have been identified as metalloenzymes (Harris et al., 2010; CAZy, 2013). Metal ion cofactors such as Mn^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, and Mg^{2+} have increased the activity of metal-activated fibrolytic enzymes (BRENDA, 2013), but to our knowledge their effects on EFE used in ruminant nutrition have not been examined.

The productivity of dairy cattle in the southeastern United States is limited by the low digestibility of tropical grasses such as bermudagrass (*Cynodon dactylon*), which is the most widely grown perennial grass in the south (Newman, 2007). Recent research identified 5 EFE that improved the NDF digestibility (**NDFD**) of bermudagrass haylage and optimized their dose rates (Romero et al., 2015a,b). Whether adding COF to these EFE would synergistically increase their hydrolytic effects is unknown.

The current study is the third of a series of 4 studies aimed at strategic development of EFE that would increase the performance of dairy cows fed tropical forage diets. The first study tested effects of 12 EFE on the preingestive hydrolysis, NDFD, and fermentation of bermudagrass haylage, identified the 5 most-promising EFE, and characterized proteomic differences between the most- and least-effective EFE (Romero et al., 2015a). The second study determined the optimal dose rates of the 5 EFE (Romero et al., 2015b). The final study examined effects of adding the best EFE and dose combination identified in previous studies on the performance of dairy cows (Romero et al., 2014). The objectives of the present study were to examine if adding COF to EFE would increase the beneficial effects of the EFE on the preingestive hydrolysis and in vitro digestibility and fermentation of bermudagrass haylage. The hypothesis was that adding key COF to the EFE would increase their preingestive hydrolytic effects. Furthermore, adding key COF at an appropriate dose would synergistically increase the NDFD and fermentation of EFE-treated bermudagrass haylage.

MATERIALS AND METHODS

Bermudagrass Substrate

An established stand of bermudagrass cultivar Tifton 85 in Alachua County, Florida, was harvested, ensiled, dried, ground, and chemically characterized as described by Romero et al. (2015b). The bermudagrass haylage also contained 0.36 and 0.27% water-soluble calcium and magnesium, and 24.1, 64.3, and 0.17 mg/L of water-soluble iron, manganese, and cobalt, respectively, as determined by inductively coupled plasma spectrometry (Beliciu et al., 2012) after microwave digestion (CEM Corp., Matthews, NC) at the Dairy One Forage Laboratory, Ithaca, New York.

EFE

Five previously selected (Romero et al., 2015a) commercial and experimental EFE that were evaluated by Romero et al. (2015b) were used. Their enzymatic activities, protein concentrations, densities, forms, mineral concentrations, application rates, and biological sources are listed in Table 1. The EFE application rates were chosen because they had optimized the NDFD of bermudagrass haylage in a previous study (Romero et al., 2015b). The mineral concentrations of the EFE were determined using the methods of Beliciu et al. (2012).

Screening COF for Synergistic Effects on the Hydrolytic Potential of EFE (Experiment 1)

An experiment was carried out to determine the effects that each of 5 COF had on EFE hydrolysis of bermudagrass haylage cell walls before consumption by animals. The quantity of water-soluble carbohydrates (**WSC**) released during preingestive hydrolysis of bermudagrass haylage was the primary measure of the hydrolytic potential of the EFE and COF. The preingestive hydrolysis procedure described by Krueger and Adesogan (2008) was used except that the EFE were diluted in nanopure water (2 mL) instead of citrate-phosphate buffer to avoid the potential chelating effects of citrate. Metal ion cofactors used were chloride salts of divalent cations of manganese, cobalt, iron, calcium, and magnesium. These COF were chosen because they had increased the activity of fibrolytic enzymes on pure substrates (BRENDA, 2013). Units for COF application rates are given in millimolar based on the convention in enzymology (BRENDA, 2013). Each COF was added to the EFE solution to achieve a final concentration of 1 mM (Lai et al., 2009) and sodium azide was added (0.02% wt/vol) to prevent substrate degradation by microbes (Krueger and Adesogan, 2008). The EFE-COF solution was added to 50-mL tubes containing 0.5 g of bermudagrass haylage and the tubes were incubated for 24 h at 25°C in quadruplicate. For each EFE, 2 blank tubes without substrate were included to correct for contributions from the EFE. After the incubation, 15 mL of nanopure water was added and the suspension was shaken (Eberbach reciprocating shaker, model 6000, Eberbach Corporation, Ann Arbor, MI) for 1 h at 260 oscillations/min to extract water-soluble compounds, filtered through previously dried (60°C for 48 h) and weighed 125-mm Whatman