IJAS Iranian Journal of Applied Animal Science

Influence of Fibrolytic Enzymes on the *in vitro* Hydrolysis and Fermentation of Different Types of Roughages Treatment

Research Article

A. Moharrery[1*]

[1] Department of Animal Science, Faculty of Agricultur, Shahrekord University, Shahrekord, Iran

Received on: 7 Oct 2013
Revised on: 22 Oct 2013
Accepted on: 30 Oct 2013
Online Published on: Sep 2014

*Correspondence E-mail: moharrery@agr.sku.ac.ir

Online version is available on: www.ijas.ir

ABSTRACT

The effects of pre-treating different types of roughages with alkali on the efficacy of exogenous fibrolytic enzymes for improving their digestibility were studied *in vitro* in factorial arrangement 4 × 3 × 5 (enzyme, treatment and roughage types). Two fibrolytic feed enzymes novozyme (N) and celloclast (C) their combination (N+C) were evaluated for their potential to improve *in vitro* degradation of different roughages including two straws (wheat straw: WS; barley straw: BS), grass hay (GH), corn stover (CS) and corn cobs (CC) as untreated or alkaline treated (NaOH or urea). The enzyme products were in liquid form and applied at a rate of 1 mg enzyme protein/g DM substrate. Anaerobic buffer medium and strained ruminal fluid were added to the *in vitro* incubations. Degradabilities of dry matter (DM), neutral detergent fibre (NDF), cellulose, soluble protein and reducing sugar profiles were determined at the end of the 24 h incubation. Overall NDF degradability of roughages were slightly increased by alkaline treatment ($P<0.05$) but, the addition of fibrolytic enzymes directly to the ruminal fluid at the application rates could not increase significantly DM or cellulose degradation ($P>0.05$). Use of fibrolytic enzymes released higher amounts of reducing sugars and lower soluble protein compared to control ($P<0.05$). Alkali treatment increased ($P<0.05$) the degradability of DM and fiber degradability. It was higher in NaOH treated forage compared to urea treated forages ($P<0.05$).

KEY WORDS alkaline treatment, fibrolytic enzymes, *in vitro* degradation, roughages.

INTRODUCTION

Production of grains generates residues such as straw and stover, which are usually utilized in different ways. It is estimated that 2000 million tonne of straw are produced globally from cereal crops each year (FAO, 2002). The major factors limiting the use of cereal straws as ruminant feeds are their low organic matter degradability and the variable cost of the other feed ingredients. It has been accepted that alkaline treatment removes phenolic compounds and disrupts the lignin carbohydrate complexes thereby increasing accessibility of the substrate to enzymatic action. Supplementing ruminant diets with fibre degrading enzymes has been shown to improve feed utilization and animal performance (Beauchemin *et al.* 2003). However, the effectiveness of feed enzymes in ruminant diets is dependent upon substrate enzyme specificity. Thus, it is important to establish the optimum enzyme activities for the degradation of forages. In one study, the use of cellulases improved the degradation characteristics of rice straw, and further improved the nutritional value of steam-treated rice straw (Liu and Orskov, 2000). Euna *et al.* (2006) showed synergistic effect between ammonia pretreatment and fibrolytic enzyme addition for *in vitro* degradation of rice straw and Wang *et al.* (2004) reported significant interactive effects of pretreatment and enzymes for all ruminal degradation pa-

rameters. Alkali treatment also increased the rate and extent of *in situ* DM disappearance irrespective of enzymes.

The objectives of this study were to investigate whether adding enzymes to untreated or alkaline treated roughages improved DM and fibre degradation.

MATERIALS AND METHODS

Roughage type

There were five roughages, comprising of wheat straw (*Triticum aestivum*), barley straw (*Hordeum vulgare*), grass hay, corn stover (*Zea mays indentata*) and corn cobs (*Zea mays indentata*), feeds were acquired from Det Jordbrugsvidenskabelig Fakultet (DJF) farm in faculty of agricultural sciences, university of Aarhus.

Roughage treatment

Roughage was treated in loose form with urea solution (1:1) containing 5% urea (w/w) and stored for two weeks in vacuumed air-tight polyethylene bags at a temperature of 37 ˚C sodium hydroxide (NaOH) treatments were performed by soaking roughages in a 4% NaOH solution for 5 min and then kept on plastic mesh and left overnight. To remove residues on NaOH the treated straw was soaked in cold water for 15 min and finally rinsed two times in fresh cold water. However, it may also remove solubilized constituents of the forages under this type treatment. After treatment the material was oven dried (60 ˚C) and ground through a 1-mm screen for further analyses.

Enzyme treatment

The study was conducted using two commercial enzymes. One enzyme named Celluclast™ (C) was obtained from Novo Nordisk Bioindustrials, Inc.™, Danbury, Conn. Celluclast™ is a mixture of various enzymes which generally contains approximately 80% 1,4-ß-D-Glucan cellobiohydrolase, approximately 15% 1,4-ß-D-Glucan glucanohydrolase, and approximately 5% 1,4-ß-D-Glucosidase. Another enzyme was Novozyme (N) and contained mainly cellulases. The 1:1 combination of C and N enzymes (C+N) also prepared before applied to the roughages. These enzyme products were in liquid form and applied at a rate of 1 mg enzyme protein/g DM substrate. Approximately 0.5 g DM of the ground sample was weighed into glass tube in three replications. Fibrolytic enzymes were diluted in distilled water and added to the corresponding tube just before inoculating with ruminal fluid. Ruminal fluid was collected from three ruminally fistulated Holstein cows fed hay and concentrate at the maintenance level. Ruminal contents were obtained and strained through surgical gauze. The strained ruminal fluid was immediately transferred to the laboratory in a Thermos flask. In the laboratory the strained rumen fluid was mixed with a CO_2 saturated standard anaerobic buffer medium in a ratio 1:5 v/v. To each warmed tube (39 ˚C) prepared as above, 60 mL of rumen fluid and buffer mix were added and then incubated in a water bath at 39 ˚C and flushed with oxygen free CO_2. The tubes were sealed with a rubber stopper equipped with a crimp valve and were incubated for 24 h. A blank (rumen fluid+buffer) was incubated at the same time in each batch. These controls were used to correct for fermentation residues resulting directly from the inoculum. Upon removal, the tubes were placed in the ice box to stop fermentation. Then, contents of the incubation tubes were centrifuged at 4600 × *g* for 20 min. Ten milliliters of the supernatant was collected in another tube and stored frozen at −80 ˚C until analyzed. The remaining content of tube was transferred to a tared number 2 filter crucible and washed with water, then dried at 60 ˚C over night for determination of DM residue.

Chemical analysis

Samples were then prepared for the chemical composition analyses. Crude protein was analyzed by Kjeldahl method (copper catalyst), neutral detergent fiber (NDF) was determined according to the method of Mertens (2002). Cellulose was determined according to the method of Cline *et al.* (1966). Ash was determined by ignition to a constant weight at 525 ˚C using programmed furnace. The range of chemical composition for roughage samples are shown in Table 1. The total reducing sugar (R-Sugar) concentration was determined by the dinitrosalicilic (DNS) acid method described by Miller (1959). Soluble protein measured with the Folin phenol reagent according method described by Lowry *et al.* (1951).

Statistical analysis

The complete randomised model in factorial arrangement 4 × 3 × 5 (enzyme, treatment and roughage types) was used to analyse data for DM, NDF or cellulose degradability at each time of incubation. The data were analysed using the general linear model procedure of SAS (2009).

RESULTS AND DISCUSSION

Effect of enzyme application on DM and fiber degradation is shown in Table 2. It seems that when enzyme was applied directly to inoculants media (no pretreatment) no improvement was obtained in DM or fiber degradation. Pretreatment enzyme application was not successful for for NaOH treated and urea treated roughages. Application of enzyme to form of pretreatment for NaOH treatment showed no improvement (data is not showed), because the pH value in this forage was about 10.0, and at this pH cellulase activity is quite low. This reason, it seems for these types of forages enzyme application should be done after neutralized them.

Table 1 Range of chemical composition (%) of roughage on DM basis

Roughage	Crude protein	Neutral detergent fiber	Cellulose	Ash	Organic matter
Untreated					
Wheat straw	4.00	77.14	39.85	3.76	96.24
Barley straw	6.44	74.98	37.10	4.55	95.45
Grass hay	7.88	56.49	26.67	3.82	96.18
Corn stover	4.25	77.94	41.96	2.38	97.62
Corn cobs	3.75	77.32	33.52	1.55	98.45
NaOH treated					
Wheat straw	2.88	75.27	47.28	5.58	94.42
Barley straw	3.81	74.96	51.56	6.14	93.86
Grass hay	6.88	59.94	34.19	11.41	88.59
Corn stover	3.00	70.15	47.03	12.78	87.22
Corn cobs	2.31	75.14	37.17	5.86	94.14
Urea treated					
Wheat straw	9.06	77.87	39.09	3.93	96.07
Barley straw	9.50	74.44	37.04	4.96	95.04
Grass hay	18.94	56.32	26.36	4.04	95.96
Corn stover	7.31	75.69	42.85	2.56	97.44
Corn cobs	14.94	77.81	33.57	1.54	98.46
Treat means*					
Untreated	5.26^{b}	72.77	35.82	3.21^{b}	96.79^{a}
NaOH treat	3.78^{b}	71.09	43.45	8.36^{a}	91.64^{b}
Urea treat	11.95^{a}	72.43	35.78	3.41^{b}	96.59^{a}
SE	1.413	3.749	2.942	1.008	1.008

* All data from chemical composition of roughage (wheat and barley straw, grass hay, corn stover and corn cobs was pooled and each number indicated overall means for each constituents of roughage under different treatments.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SE: standard error and DM: dry matter.

Table 2 Effect of enzyme on different forage treatment

composition	Enzyme				SE	P-value
	Control	Novozyme	Celluclast	N + C		Enzyme × Forage
Untreated forages						
DM	33.6^{ab}	34.0^{a}	34.0^{a}	32.3^{b}	0.510	0.9854
NDF	24.1	24.9	25.2	23.4	0.747	0.8806
Cellulose	25.4^{a}	20.1^{b}	20.7^{b}	18.6^{b}	1.245	0.9851
NaOH treated forages						
DM	41.4	41.9	41.6	42.1	0.193	0.1987
NDF	33.0^{b}	34.7^{a}	34.8^{a}	34.6^{a}	0.577	0.6980
Cellulose	27.6^{a}	26.2^{b}	26.0^{b}	24.8^{c}	0.494	0.9097
Urea treated forages						
DM	40.4^{ab}	41.4^{a}	38.9^{b}	39.0^{b}	0.466	0.2371
NDF	31.6	34.0	32.1	31.5	0.908	0.5084
Cellulose	33.1^{a}	30.4^{b}	28.4^{b}	28.3^{b}	0.886	0.5674

The means within the same raw with at least one common letter, do not have significant difference (P>0.05).
N + C: novozyme + celluclast.
SE: standard error.
P: probability.
DM: dry matter and NDF: neutral detergent fiber.

Urea treated forages have a mean pH 6.00 (5.8-6.44) which it was not a limiting factor for pretreatment with enzyme but, remaining urea in the forage after treatment was the factor for denaturation of cellulase. The proportion of urea-N retained by the straw ranged from 33% in materials containing 10% unhydrolysed urea to 22% in samples in which hydrolysis was complete (Taiwo *et al.* 1995).

Variable nitrogen retention values have been reported in studies with urea treatment. Williams *et al.* (1984) obtained 36% nitrogen retention while Dias Da Silva *et al.* (1988), got 57%. They used 60 and 70 g urea per kg DM, 300 and 400 g moisture per kg, 40 and 54 day treatment period and 18 and 22 °C temperature, respectively. Urea is a Chaotropic agent (Arakawa and Timasheff, 1984), which has the potential for denaturing proteins like cellulase (Turner *et al.* 2003). Pretreatment of forage, which is treated later with urea, would increase the risk of partial denaturation of enzyme. The pretest of urea treated forage showed application of enzyme to form of pretreatment (24 h) could not improve degradability of forage (data not showed). There

was no difference (P>0.05) for the interaction between roughage treatment and type of enzyme application in all chemical composition.

Both DM and fiber degradation were not altered by enzyme level during 24 h of incubation (P>0.05). However, NDF degradability in NaOH treatments was greater (P<0.05) for the N type of enzyme. Part of this response refers to removing phenolic compounds and disrupting the lignin carbohydrate complexes by the action of high NaOH alkaline treatment thereby increasing accessibility of the substrate to enzymatic action. On the other hand, the action of cellulolytic enzymes on fibrous materials occurred during the first 10 h of incubation (Moharrery *et al.* 2009), then their action gradually was reduced, and at 24 h of incubation the enzyme activity had more or less the same activity as whatever can detect in ruminal fluid without supplemented by enzyme. In this regard, the fiber which is solubilized in short time by the action of enzyme, would probably also be easily degraded *in vivo* and therefore *in vivo* NDF digestibility would probably not be affected by a similar treatment. The present finding is in agreement with Wang *et al.* (2004) who indicated no significant differences between enzyme treated forage and control after 30 h incubation time (P>0.05) but, the difference between two types of treatment was significant after 4 h incubation.

Results of forages DM and fiber degradability in 24 h of incubation irrespective of enzyme or treatment action are shown in Figure 1. As it was expected, the grass hay showed superiority than other forages but within other low quality forages, barley straw showed higher degradability than others, followed by corn stover and wheat straw and corn cobs, which ranked the lowest. Tuah and Orskov, (1987) indicated that the hemicellulose content of the corn cobs, was very high (46.4%). Hemicellulose is more closely associated with lignin than any other polysaccharide fraction and it is believed to be bound to phenolic constituents (Van Soest, 1982). The cellulose and the hemicellulose contained in corn cobs may therefore not be made readily available for microbial degradation, thus decreasing its DM and fiber degradability (Tuah and Orskov, 1987).

Alkali treatment increased (P<0.05) DM and fiber degradability (Figure 2). It was higher in NaOH treated forage compared to urea treated forages (P<0.05). The cellulose degradability was highest for urea treatment roughages followed the NaOH treatment and untreated roughages (Figure 2). Beside of some difficulty in relation to alkaline treatment of forages, results of present experiment showed that alkaline treatment affected stronger than enzyme application for DM and fiber degradability.

Reducing sugar and soluble protein in the inoculant media after 24 h of incubation showed that enzyme application could release higher and significant (P<0.05) amounts of reducing sugars than control (Figure 3). Rumen microbes in the inoculant media used these sugars and in this process, they used protein, which was available in the media. For this reason, the protein concentration in the media was significantly (P<0.05) reduced when enzymes were applied to the forages (Figure 4).

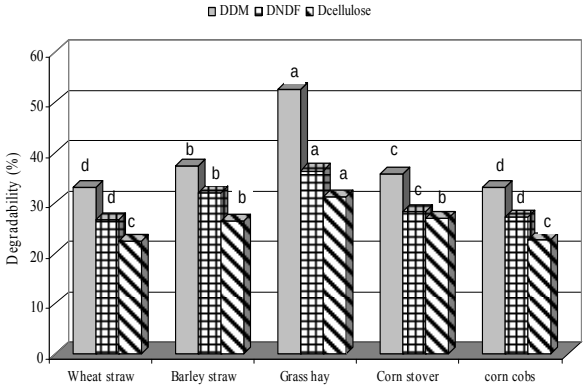

Figure 1 The degradability of different forages after 24 h of incubation. Means with the same letter on same color bar are not significantly different (P<0.05)

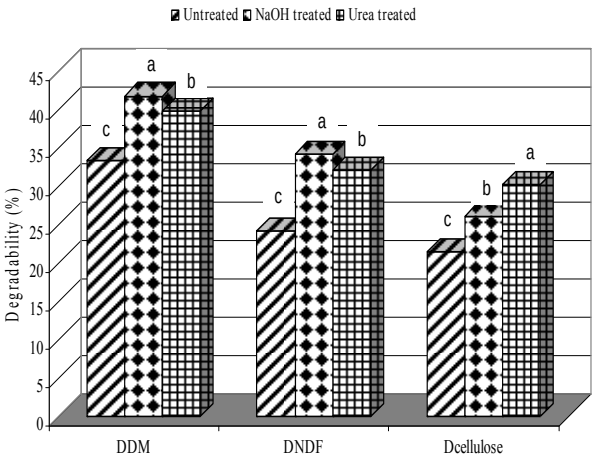

Figure 2 Effects of different alkaline treatment on forages degradability after 24 h of incubation
Means with the same letter on same color bar are not significantly different (P<0.05)
DDM: dry matter degradability; DNDF: NDF degradability; D-cellulose: cellulose degradability

It was expected that with action of enzyme on forages cellulose, sugars as the end products of this hydrolyzing action were releases into the media. The present finding is in agreement with Lee *et al.* (2000) who reported that strong correlation coefficient (92.37%) between cell wall digestion and reducing sugar contents in the culture supernatant, suggesting that reducing sugars were released from the cell wall material by microbial degradation. Higher releasing of reducing sugars to the media resulted to better synchronization between sugar as an energy source and

available nitrogen source for microbial growth and reduction of soluble nitrogen in the media. In this manner, it could be expected to lower concentrations of soluble protein in the media, which contained fibrolytic enzymes.

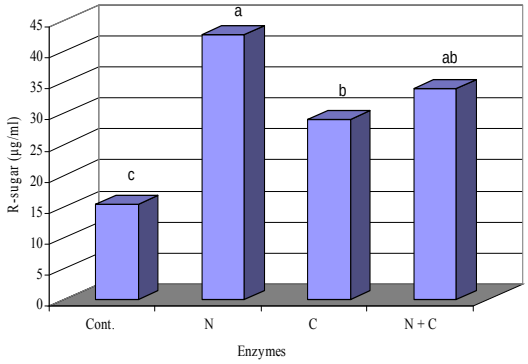

Figure 3 The reducing sugar (µg R-sugar/mL/g DM) in the tube contents after 24 h of incubation on different type of enzymes
Means with the same letter on each bar are not significantly different (P<0.05)
N: novozyme; C: celluclast; N + C: novozyme + celluclast

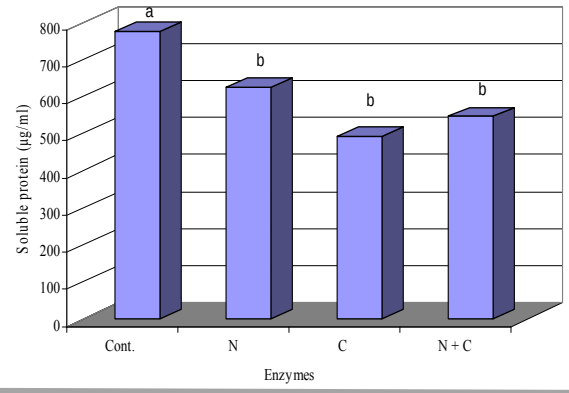

Figure 4 The soluble protein (µg/mL/g DM) in the tube contents after 24 h of incubation on different type of enzymes
Means with the same letter on each bar are not significantly different (P<0.05)
Cont: control; N: novozyme; C: celluclast; N + C: novozyme + celluclast

CONCLUSION

The low nutritive value of roughages material for ruminants has been demonstrated in numerous studies. The present study showed that alkali treatment enhanced *in vitro* degradation of roughages material rather than direct application of fibrolytic enzymes, but NaOH pretreatment was more effective than urea treatment. The effectiveness of fibrolytic enzymes was enhanced when they were used with NaOH treatment roughages material. Treatment with NaOH, urea and cellulase enzymes resulted in different extensive degradation of roughages in the media. This indicates that the action of enzymes basically differs from alkali treatment on roughages. The fact that fibrolytic enzymes were able to release higher reducing sugars, suggested that direct application of enzyme also could improve fiber degradability of roughages. It seems that higher alkalinity of NaOH treated material and remaining of urea in the roughages material after treatment by urea were the factors that inactivated fibrolytic enzymes. Any action to eliminate these problems can improve degradability of fibrous material by the enzyme action.

ACKNOWLEDGEMENT

The author would like to express his gratitude to the Faculty of Agricultural Science, University of Aarhus, for hosting his sabbatical.

REFERENCES

Arakawa T. and Timasheff S.N. (1984). Protein stabilization and destabilization by guanidinium salts. *Biochemistry*. **23,** 5924-5929.

Beauchemin K.A., Colombatto D., Morgavi D.P. and Yang W.Z. (2003). Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *J. Anim. Sci.* **81,** 37-47.

Cline T.R., Garrigus U.S. and Hatfield E.E. (1966). Addition of branched and straight chain volatile fatty acids to purified lamb diets and effects on utilization of certain dietary components. *J. Anim. Sci.* **25,** 734-739.

Dias Da Silva A., Mascarenhas Ferreira A. and Guedes C.V.M. (1988). Effects of moisture level, treatment time and soyabean addition on the nutritive value of urea treated maize stover. *Anim. Feed Sci. Technol.* **19,** 67-77.

Euna J.S. Beauchemin K.A. Hong S.H. and Bauer M.W. (2006). Exogenous enzymes added to untreated or ammoniated rice straw: Effects on *in vitro* fermentation characteristics and degradability. *Anim. Feed Sci. Technol.* **131,** 86-101.

FAO. (2002). Agricultural Statistics. Food and Agriculture Organization of the United Nations, Rome. http://apps1.fao.org.

Lee S.S., Ha J.K. and Cheng K.J. (2000). Relative contributions of bacteria, protozoa, and fungi to *in vitro* degradation of orchard grass cell walls and their interactions. *Appl. Environ. Microb.* **66,** 3807-3813.

Liu J.X. and Orskov E.R. (2000). Cellulase treatment of untreated and steam pre-treated rice straw effect on *in vitro* fermentation characteristics. *Anim. Feed Sci. Technol.* **88,** 189-200.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.

Mertens D.R. (2002). Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. *J. AOAC. Int.* **85,** 1217-1240.

Miller G.L. (1959). Use of dinitrosalicilic acid reagent for determination of reducing sugar. *Anal. Chem.* **31,** 426-428.

Moharrery A., Hvelplund T. and Weisbjerg M.R. (2009). Effect of

forage type, harvesting time and exogenous enzyme application on degradation characteristics measured using *in vitro* technique. *Anim. Feed Sci. Technol.* **153,** 178-192.

SAS Institute. (2009). User's Guide: Statistics, Version 9.2 Edition. SAS Inst., Inc., Cary, NC, USA.

Taiwo A.A., Adebowale E.A., Greenhalgh J.F.D. and Akinsoyinu A.O. (1995). Techniques for trapping ammonia generated from urea treatment of barley straw. *Anim. Feed Sci. Technol.* **56,** 133-141.

Tuah A.K. and Ørskov E.R. (1987). The degradation of untreated and treated maize cobs and cocoa pod husks in the rumen. Pp 363-378 in: Overcoming Constraints to the Efficient Utilization of Agricultural by-Products as Animal Feed. International Livestock Research Institute, Ed. ILRI (aka ILCA and ILRAD).

Turner M.B., Spear S.K., Huddleston J.G., Holbrey J.D. and Rogers R.D. (2003). Ionic liquid salt induced inactivation and unfolding of cellulose from *Trichoderma reesei. Green Chem.* **5,** 443-447.

Van Soest P.J. (1982). Nutritional Ecology of the Ruminant. Corvallis, Oregon.

Wang Y., Spratling B.M., Wiedmeier D.R. and McAllister T.A. (2004). Effect of alkali pretreatment of wheat straw on the efficacy of exogenous fibrolytic enzymes. *J. Anim. Sci.* **82,** 198-208.

Williams P.E.V., Innes G.M. and Brewer A. (1984). Ammonia treatment of straw via the hydrolysis of urea (II). Addition of soyabean (urease), sodium hydroxide and molasses: effects on the digestibility of urea treated straw. *Anim. Feed Sci. Technol.* **11,** 115-124.