Emir. J. Agric. Sci. 2003. 15 (1) : 76-83
http://www.cfs.uaeu.ac.ae/research/ejas.html

Incorporation of isoacids, oil, NPN and protein in the ration of sheep and their effects on protease and amylase in the rumen fluid

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Abstract: Five total mixed rations were prepared from ragi [finger millet (*Eleusine Coracana)*] straw as a roughage (48%) and mixed concentrate (52%), supplemented with 1% isoacid (I-C_4, I-C_5, C_5 and phenylacetic acid in equal proportions) or oil (ground nut oil, 5% more than control) or urea (5% more crude protein than control), and protein (ground nut cake, 5% crude protein more than control). Diets were fed to sheep in 5×5 Latin square design. Biochemical activities were estimated for enzymes e.g., protease and amylase in various fractions of rumen fluid. Rumen samples were taken 3-4 hours after feeding and mixed rumen bacteria were separated as a strained rumen fluid without protozoa (SRFWP), cell free rumen fluid (CFRF) and enzymes associated with the bacteria cell (EABC). Samples of SRFWP and EACB contained higher enzyme activity than CFRF, but individual enzymes in each fraction showed different activities. Except for protein diet in amylase activity. EACB fraction, other fractions. No significant difference was observed between diets.

Key word: enzymes, rumen, isoacids, urea, and sheep.

إضافة الأيسواسيد والزيت والبروتين غير الحقيقي والبروتين في عليقة الأغنام وتأثيراته على إنزيم البروتياز والإميلاز في سائل الكرش

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الملخص : خمسة علائق تم تحضيرها من تبن الدخن الإصبعي بنسبة 48% مع علف مركز 52% مع إضافة إما 1% ايسواسيد (I-C_4,I-C_5,C_5 and Phenylacetic acid بنسبة متساوية) أو زيت (5% زيت من اللوز المطحون أكثر من الشاهد) أو يوريا (5% بروتين خام أكثر من الشاهد) أو بروتين حقيقي (5% بروتين من كسبة اللوز المطحون أكثر من الشاهد). تم إعطاء هذه العلائق لخمسة أغنام بتصميم 5×5 المربع اللاتيني. أخذت القياسات الكيميائية لإنزيم البروتياز والإميلاز من سائل الكرش. وقد تم أخذ عينات سائل الكرش 3-4 ساعات بعد التغذية وقد تم عزل خليط من بكتريا الكرش بدون البروتوزوا وسائل الكرش خالي من الخلايا البكتيرية وسائل الكرش المحتوى على الإنزيمات ذات العلاقة بخلايا البكتريا. احتوت عينات سائل الكرش المحتوى علي خليط من بكتريا الكرش بدون البروتوز والأعلى نسبة أعلى من نشاط الأنزيمات مقارنة بسائل الكرش المحتوى علي الأنزيمات ذات العلاقة بخلايا البكتريا. سائل الكرش الخالي من الخلايا البكتيرية ولكن كل إنزيم اختلف في نشاطه عن عينات سائل الكرش المختلفة.لم يكن هناك أى تأثير للعلائق على نشاط الإميلاز في عينات سائل الكرش فيما عدا سائل الكرش المحتوى على الإنزيمات ذات العلاقة بخلايا البكتريا فى عليقة البروتين.

كلمات مفتاحية: إنزيمات، كرش ، أيسواسيد، يوريا، أغنام

Introduction

The complexity of the digestive process in ruminants is well recognized by all who work with ration balancing in those species. Digestion is a two step process, first by microbes in the rumen, and secondly by the host animal in the lower gut. Therefore to understand the utilization of feedstuffs, we need to consider two entirely separate but interdependent ecosystems. The rumen is the first and largest compartment inhabited by microflora and microfauna. The ruminal microbes include a large variety of bacterial, protozoal and fungal species making the rumen habitat quite complex. These microbes through their enzymes (Pathak *et al.,* 1996) play the roles in digestive processes. Considerable progress has been made toward development of quantitative relationships among the chemical composition of ruminant feeds,

dynamic aspects of digestion in the rumen, products of digestion absorbed by the ruminant, and, most important, how these can be manipulated to improve animal productivity (Dehority and Orpin, 1988; Dehority, 1998).

The specific activity of the bacterial proteinase was 7 to 10 times higher than that of separated protozoa when either whole cells or cell extracts were assayed (Brock *et al.*, 1982). Nugent and Mangan, (1981) have reported that the protozoal population made up less than 10% of the proteolytic activity of whole rumen fluid. Most of the protease is cell-bound (Prins *et al*., 1983; Nugent and Mangan, 1981), and only a small portion is extracellular (Agarwal *et al.,* 1991). The major proteolytic bacteria in the rumen are starch and hemicellulose degrading bacteria (Hazlewood *et al*., 1981; Wallace and Brammal, 1985).

Amylolytic bacteria are one of the major groups of bacteria present in the rumen on any diet, but the number may increase ten fold on high starch diets (Kamra and Pathak, 1996). The quality of enzyme varies depending upon the substrate present (Cotta & Whitehead, 1993).

The objectives of the present experiment were to study the effects of isoacids, oil, urea and protein on protease and amylase activities in rumen fluid.

Materials and methods

Animals

Five rams between 18 and 24 months of age and body weight (between 23 – 26 kg) (24 ± 2.1) were selected for the experiment.

Fodder

Sun dried, good quality ragi [finger millet (Eleusine Coracana)] straw was chopped with help of a handchopper into a size of about five to six centimeters. Straw formed 48% of rations.

Feed

The ingredients used in the preparation of the total mixed rations (TMRs) are shown in Table 1. The experimental diets consisted of a control diet, control diet plus one percent mixed isoacids (i-C_4, i-C_5, C_5 and phenylacetic acid in equal proportions), oil diet (groundnut oil, 5% more ether extract than control diet), a urea diet (5% more crude protein from urea than control diet) and a protein diet (5% more crude protein from groundnut cake than control diet).

Management of feeding and duration:

The amount of feed offered in the adaptation period to each sheep was calculated according to its live weight to give near maintenance level. In this manner, the amount of feed offered was 625 g air dried TMR (Total Mixed Ration) per day for each sheep. The diets were fed twice daily at 0600 and 1500 hours. Clean good water was given ad libitum after feeding for ½ hour and after this time water was removed.

Duration of each period was 15 days, with 14 days of adaptation and 1 day of sampling. After this time the ration of each sheep was changed until each sheep had received all five rations.

Sampling of rumen contents

At 15-days intervals, rumen samples from each animal were removed via a stomach tube with the help of a vacuum pump 3 to 4 hr after morning feeding. Conical flasks for collection of samples were kept in an ice bath, and approximately 150 ml of all samples were transferred immediately to the laboratory. Rumen fluid along with some particles was strained through cotton gauze and centrifuged at 1000 × g for 10 min.

Table 1. Composition % of experimental treatment

	Control	Isoacid	Oil	Urea	Protein
Ingredient Item					
Ragi straw	48.00	48.00	48.00	48.00	48.00
Maize	20.80	20.80	15.60	20.80	13.52
Groundnut Cake	5.20	5.20	6.24	5.20	24.44
Rice bran	13.52	13.52	11.44	13.52	3.64
Soybean meal	7.28	7.28	8.32	5.20	5.20
Oil (Groundnut)	-	-	5.20	-	-
Urea	-	-	-	2.057	-
Isoacid [1]	-	1.04	-	-	-
Vitamin + Mineral supplement	1.04	1.04	1.04	1.04	1.04
Calcite	1.04	1.04	1.04	1.04	1.04
Salt	1.04	-	1.04	1.04	1.04
Dicalcium phosphate	2.08	2.08	2.08	2.08	2.08
Composition (DM basis)					
DM(%)	84.53	84.53	85.05	84.76	84.38
ME (Mcal/kg) 2	2.79	2.79	2.96	2.72	2.76
TDN (%)a	69.87	69.87	75.24	67.96	68.73
CP (%)	12.25	12.25	12.36	17.68	17.11
DIP(%)a	8.89	8.89	9.02	14.82	13.08
UIP (%)a	3.37	3.37	3.33	3.05	4.04
EE (%)	2.34	2.34	7.58	2.31	1.55
NFE (%)	54.29	54.29	49.47	53.34	50.02
Ash (%)	12.28	12.28	12.07	12.11	12.18
CF (%)	20.35	20.35	19.73	20.10	20.35
ADF (%)	26.23	26.33	25.42	25.91	26.23
Cellulose (%)	20.15	20.15	19.82	19.84	21.01
Ca (%)a	1.13	1.13	1.13	1.13	1.13
P (%)a	0.92	0.92	0.87	0.90	0.77

[1] Isoacids included of I-C4, I-C5, C5 and phenylacetic acid in equal proportions.
[2] From nutrient requirements of sheep (NRC, 1985). Only data related to ragi straw has been taken from Ranjhan, 1993.

The supernatant fluid was carefully decanted and divided into two parts. One part was stored in –196 °C in liquid nitrogen for future analysis (Moharrery and Das, 1998a) and was designated strained rumen fluid without protozoa (SRFWP). The other part (25 ml) was centrifuged again (26000 × g, 15 min) and supernatant fluid was carefully decanted and stored in –196 °C and designated cell free rumen fluid (CFRF). The resulting pellet was resuspended in buffer solution (Moharrery, 1999) and this suspension was centrifuged (26000 × g, 10 min). After decanting the supernatant fluid, the pellet was resuspended to one fifth of the original volume in buffer solution and cells were broken by five 60-second periods of sonication with 60-s intervals under ice bath conditions. Sonically-disrupted cells were centrifuged at 40,000×g for 10 min. The clear supernatant was stored at –196 C^{o} and designated enzyme associated with cell envelope (EACB).

Determination of rumen enzymes

The assay procedure for protease was based on the method of Blackburn (1968). A unit of proteolytic activity was defined as the amount of enzyme, which would solubilize the equivalent of 1.0 μg tyrosine in 1 min.

The assay procedure for amylase was based on Pathak *et al.* (1996). The enzyme attacks α- 1,4 glucan linkages of starch and glycogen and releases maltose, isomaltose, larger oligosaccharides and glucose. The α-amylase activity is determined colorimetrically by measuring the rate of release of reducing sugar during the incubation of enzyme with starch solution (1%). Enzyme activity was expressed as μg reducing sugars (glucose) released per min per ml sample. All enzyme activity measurements were performed at least in duplicate.

Statistical analysis

This experiment was designed as a 5×5 Latin square. All data from each variable were analyzed with the GLM procedures of SAS (1987). When significant ($P< 0.15$) effects were detected, means were separated by a Duncan test.

Results

Estimation of enzyme activity in SRFWP

The effect of the different treatments on enzyme activity on rumen has been presented in Table 2. No significant difference was detected between treatments for protease activities ($P >0.05$). Amylolytic activity is shown in Table 2. Highest value was on the urea treatment and the lowest value was on the protein diet. This difference was significant ($P < 0.05$). The other treatments were also significantly different from the protein treatment ($P < 0.05$). Energy also in the form of oil did not affect amylolytic activity.

Assay of enzyme activity in CFRF

After thawing the CFRF samples from –196 ^{o}C to room temperature, estimation of enzyme activity was performed (Table 3) Protease activity is associated with cell and in the CFRF. This assay evaluated non-cell-associated protease, which was secreted from cells.

The protein diet showed the highest value of proteolytic activity compared with other treatments ($P < 0.05$) (Table 3). Crude protein in the form of urea did not affect proteolytic activity ($P > 0.05$).

Table 2. Influence of different treatments on enzyme activity in SRFWP

Treatment	Protease (Unit/ml)	Amylase (μg gluc./min./ml)
Control	0.201 (0.078)	172.2[a] (45.9)
Isoacid	0.186 (0.038)	161.6[a] (13.6)
Oil	0.152 (0.050)	156.5[a] (25.1)
Urea	0.161 (0.041)	181.6[a] (37.1)
Protein	0.162 (0.029)	90.1[b] (17.0)
SE	0.007	4.67

[1]: Means (standard deviation);
[2]: Strained rumen fluid without protozoa; SE: Standard Error
a, b, Means with same letter in each column are not significantly different ($P<0.05$).

In general, amylase activity in CFRF is 65.4% less than SRFWP. Table 3 has shown highest amylolytic activity for isoacid and lowest value for oil treatment ($P < 0.05$). The oil treatment caused about 40% depression in CFRF amylase activity ($P < 0.05$). Crude protein in the form of groundnut cake, also reduced amylase as much as 26.3% compared with control ($P < 0.05$). Urea also did not change amylolytic activity compared with control ($P < 0.05$).

Assay of enzyme activity in EACB

EACB as a part of rumen fractions was found to have greater hydrolytic activity than CFRF (Table 3 and 4). Enzyme activity in this fraction was compatible to SRFWP, though no live cells were found generally, proteolytic activity in EACB was 2.3 time more than in CFRF. The greatest activity was on the protein diet and the lowest value was on the isoacid diet. Difference between highest and lowest values was 20.5%, but was not significant ($P> 0.05$).

Amylolytic activity in EACB is presented in Table 4. A significant difference was seen between the isoacid and protein diets ($P<0.05$). Numerical differences between other diets were large, but due to high variability in the data, these differences were not significant ($P> 0.05$).

Table 3. Influence of different treatments on enzyme activity in CFRF

Treatment	Protease (Unit/ml)	Amylase (μg gluc./min./ml)
Control	0.090^{b} (0.027)	60.05^{a} (10.96)
Isoacid	0.085^{b} (0.014)	63.11^{a} (7.12)
Oil	0.074^{b} (0.015)	36.64^{b} (1.07)
Urea	0.077^{b} (0.015)	61.07^{a} (10.89)
Protein	0.164^{a} (0.043)	44.28^{b} (24.89)
SE	0.003	1.97

Means: standard deviation; : Strained rumen fluid without protozoa;
SE: Standard Error; a,b,: Means with same letter in each column are not significantly different.

Table 4. Influence of different treatments on enzyme activity in EACB

Treatment	Protease (Unit / ml)	Amylase (μg gluc./min./ml)
Control	0.220 (0.046)	208.7^{ab} (97.0)
Isoacid	0.204 (0.092)	102.4^{b} (48.8)
Oil	0.233 (0.067)	194.7^{ab} (127.2)
Urea	0.216 (0.051)	244.1^{ab} (115.7)
Protein	0.245 (0.082)	290.1^{a} (19.0)
SE	0.007	19.62

Means: standard deviation; Strained rumen fluid without protozoa,
SE: Standard Error; Means with same letter in each column are not significantly different.

Discussion

Most of the protease is cell-bound (Prins *et al.,* 1983; Nugent & Mangan, 1981), and only a small portion (about 10-15%) is extracellular (Agrawal *et al.,* 1991). In this regard, protease activity in SRFWP and EACB was higher than CFRF. Protease activity in CFRF was half of SRFWP. The observation that the SRFWP and EACB have similar activity is in agreement with results of Cotta and Hespell, (1986). They reported that both the cell-associated and culture fluid activities had similar properties with respect to temperature stability and optimal pH. However, Blackburn and Hobson, (1960) reported that the proteolytic activity of whole rumen fluid of sheep did not depend on the diet of the animals. Proteolytic activity in CFRF was less than SRFWP and EACB, but there was a significant difference between treatments in CFRF. Five percent more crude protein than control in the form of groundnut cake resulted in 82.3% more proteolytic activity than control ($P< 0.05$) in CFRF fraction. This result showed that increasing protein could be an enhancing factor for more protease activity in cell free fraction in rumen liquor. The protein in groundnut cake is more than 70% ruminally degradable (Krishnamoorthy *et al.,* 1995) and after consumption of groundnut cake, a lot of protein will be dissolved in rumen fluid, and proteolytic bacteria have an opportunity to degrade it. In this regard, more soluble protein can be an enhancing factor for proteolytic bacteria to produce more extracellular enzyme. This effect is not related to the availability of ruminal ammonia, because the urea diet did not show more proteolytic activity than control. Results showed that about 50% of protease were extracellular enzymes. This result is in agreement with Pollock (1962). Blackburn (1968) showed that extracellular protease released to medium was 20% while Agrawal *et al.* (1991) reported that this amount is 10-15%. With providing substrate for proteolytic bacteria, their growth would be enhanced and total mass of these type bacteria could be increased. This was reported earlier by Moharrery and Das (1998b). On the other hand, proteolytic bacteria have cell-associated protease which is released to buffer after sonication. For this reason, more proteolytic activity was seen in protein diet rather than other treatments in EACB, though this difference was not significant ($P> 0.05$).

Results from Table 2 and 3 showed mean values of amylolytic activity in all diets in CFRF that were approximately 30% of the activity in SRFWP. In SRFWP, with the exception of the protein diet, other treatments had similar values in amylolytic activity, though urea treatments were higher than the others were. These results are in agreement with Slyter *et al.* (1968) who found urea-fed steers contained a greater concentration of amylolytic bacteria. However in the present experiment the protein diet had half the amylolytic activity of the urea treatment and this difference was significant ($P< 0.05$). The reasons for this were not clear although in the latter case, maize grain which provided starch in the rations, in protein and oil diets was 7.3 and 5.2 less than control in the protein and oil diets, respectively. For this reason specific amylolytic bacteria in rumen fluid on the protein diet may not have had enough substrate, and this treatment showed lowest activity among all diets. In this regard amylolytic activity in CFRF for the protein diets was also the lowest of all diets. Values in CFRF are a reflection of extracellular enzymes. When substrate for amylase activity is less in whole rumen fluid, existence of extracellular enzyme also remained at a low level; therefore the net result of this sequence gave lower activity in cell free fraction.

In the EACB fraction the situation was different. Generally, proteolytic bacteria such as *B. amylophilus* and *B.ruminicola* (Hobson & Wallace, 1982) and *S. bovis* (Wallace & Brammal, 1985) included starch hydrolyzing bacteria. More than 50% of amylolytic activity is cell bound in *S. bovis*, when starch is the substrate (Cotta, 1988). On the other hand, the protein diet is an enhancing factor for growth

of these types of bacteria. Since these bacteria have amylolytic and proteolytic activities together, after sonication, enzyme with cell envelope will release in the buffer. Therefore, amylase as well as protease will release in medium and show activity. In the present study, the net result of releasing of these enzymes indicates the highest amylase and protease activities in EACB fraction of the protein diet ($P< 0.05$). The highly correlation coefficient between amylase and protease activity reported by Moharrery (1999) is in agreement with this result.

Conclusion

Any attempt to improve ruminant nutrition must take into account the fact that alteration in feed composition will lead to a change in the ruminal ecosystem, and this will invariably result in alteration of digestibility of feed material in whole tract. In this regard oil or ether extract at a level of 7% of dry matter at maintenance level did not show negative effects on amylase and protease activities. The isoacid at the level used in this study could not show isoacid as an enhancing factor for proteolytic or amylolytic bacteria. Crude protein from urea and groundnut cake showed that they have different effects on protease and amylase activities in various fractions of rumen fluid.

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