Distribution and Activities of Hydrolytic Enzymes in the Rumen Compartments of Hereford Bulls Fed Alfalfa Based Diet

S. S. Lee[1], C.-H. Kim[2], J. K. Ha, Y. H. Moon[3], N. J. Choi and K.-J. Cheng[4]
School of Agricultural Biotechnology, Seoul National University, Suweon 441-744, Korea

ABSTRACT : The distribution and activities of hydrolytic enzymes (cellulolytic, hemicellulolytic, pectinolytic and others) in the rumen compartments of Hereford bulls fed 100% alfalfa hay based diets were evaluated. The alfalfa proportion in the diet was gradually increased for two weeks. Whole rumen contents were processed into four fractions: Rumen contents including both the liquid and solid fractions were homogenized and centrifuged, and the supernatant was assayed for enzymes located in whole rumen contents (WRE); rumen contents were centrifuged and the supernatant was assayed for enzymes located in rumen fluids (RFE); feed particles in rumen contents were separated manually, washed with buffer, resuspended in an equal volume of buffer, homogenized and centrifuged and supernatant was assayed for enzymes associated with feed particles (FAE); and rumen microbial cell fraction was separated by centrifugation, suspended in an equal volume of buffer, sonicated and centrifuged, and the supernatant was assayed for enzymes bound with microbial cells (CBE). It was found that polysaccharide-degrading proteins such as β-1,4-D-endoglucanase, β-1,4-D-exoglucanase, xylanase and pectinase enzymes were located mainly with the cell bound (CBE) fraction. However, β-D-glucosidase, β-D-fucosidase, acetylesterase, and α-L-arabinofuranosidase were located in the rumen fluids (RFE) fraction. Protease activity distributions were 37.7, 22.1 and 40.2%, and amylase activity distributions were 51.6, 18.2 and 30.2% for the RFE, FAE and CBE fractions, respectively. These results indicated that protease is located mainly in rumen fluid and with microbial cells, whereas amylase was located mainly in the rumen fluid. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 12 : 1725-1731)***

Key Words : Rumen, Hydrolytic Enzyme, Polysaccharidases, Glycosidases, Enzyme Distributions

INTRODUCTION

The rumen is being recognized increasingly as a particularly promising source of superior polysaccharide-degrading enzymes (Lee et al., 1999). Polysaccharide breakdown in the rumen is affected by the synergistic interaction of enzymes produced by a complex microbial population. Especially, cellulase and xylanase produced by ruminal anaerobic fungi are among the most active polysaccharide-degrading enzymes described to date (Gilbert et al., 1992; Trinci et al., 1994), and the activity of xylanase is 2-5 times higher than that of cellulase (Lee et al., 1995).

The rumen has a complex structure and the contents are heterogeneous, consisting primarily of a microbial suspension in free liquid, a solid mass of digesta, and a gas phase (Cheng and McAllister, 1988). Two major groups of organisms have been recognized i.e. the unattached rumen fluid (liquid phase) organisms and the adherent (particle-associated) population. Willams and Strachan (1984) monitored the location and level of activity of polysaccharide-degrading enzymes in microbial populations isolated from the liquid and particulate phases of rumen digesta. Although activity was detectable in all populations examined, it was apparent that the enzymes involved in the degradation of plant structural polysaccharides were most active in the adherent particle-associated microorganisms, whereas soluble saccharides were metabolized by the liquid phase and non-adherent populations (Williams and Strachan, 1984; Williams et al., 1984). Hydrolytic enzymes, especially cell-wall degrading enzymes from the important degrading microorganisms in the rumen microorganisms have been extensively studied, but there is less clear information at the rumen level.

The present study was conducted to determine the distributions of hydrolytic enzymes in the rumen compartments, identifying the hydrolytic enzyme activities in the rumen of Hereford bulls fed 100% alfalfa hay diets.

MATERIALS AND METHODS

Collection of rumen contents

Rumen contents were obtained from two-ruminally fistulated Hereford bulls (Body weight about 650 kg) fed a ration consisting of 100% alfalfa hay and trace minerals and vitamins twice a day (06:00 and 16:00 h). The alfalfa proportion in the diet was gradually increased for two

* Corresponding Author: J. K. Ha. Tel: +82-31-290-2348, Fax: +82-31-295-7875, E-mail: jongha@snu.ac.kr
[1] Department of Dairy Science, Gyeongsang National University, Chinju 660-701, Korea.
[2] National Livestock Research Institute, Rural Development Administration, Suweon 441-350, Korea.
[3] Department of Animal Science and Biotechnology, Chinju National University, Chinju 660-758, Korea.
[4] Institute of BioAgricultural Resources, Academia Sinica, Taipei 115, Taiwan ROC.
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weeks. Two liters of rumen digesta (whole rumen contents) that included both the liquid and solid fraction were sampled from different parts of the rumen between the cranial and ventral coronary pillars 4 h after the morning feeding.

Preparations of crude enzyme solutions

Crude enzyme solutions were prepared from four fractions that included the whole rumen contents, a rumen fluid fraction, the feed particulate fraction and the cell particulate fraction. Whole rumen contents (100 mL per animal) were homogenized using an electric mixer (Brinkmann homogenizer, Model-PT 10/35, Brinkmann Instruments Co., Switzerland) for 5 min in a cold room (4°C). This step was followed by centrifugation (10,000×g for 15 min at 4°C) and the supernatant was used as a crude enzyme solution from whole rumen contents. Another aliquot of whole rumen contents (1,000 mL per animal) was passed through four layers of cheese cloth and poured into a separating funnel that had been gassed with oxygen-free CO_2. The resultant fluid was incubated at 39°C under anaerobic conditions for up to 60 min to allow small feed particles to buoy up and microbial fraction to sediment at the bottom. The small feed particles that had risen to the surface were carefully collected by a vacuum tube, and the residue in the lower portion was then also collected under anaerobic conditions. Large feed particles retained by the cheese cloth plus small feed particles that had risen to the surface were mixed and suspended in the same volume (final volume 500 mL) of 0.5 M sodium phosphate buffer (pH 6.8). The sample was processed according to a modification of the procedure of Merry and McAllan (1983). Briefly, the material was washed by manual shaking for 5 min in a bottle with sodium phosphate buffer pre-warmed at 39°C in order to remove the non-adherent population associated with the feed particles. The sample was then centrifuged at 1,000×g, for 10 min at 4°C to recover a pellet of feed particle. The recovered particulate pellet was resuspended in the same volume (final volume 500 mL) of 0.5 M sodium phosphate buffer (pH 6.8) and then homogenized and centrifuged (10,000×g for 30 min at 4°C). The supernatant was used as the crude enzyme solutions for the analysis of feed associated enzymes (FAE, feed associated enzymes). The remaining liquid portion, cleared of small feed particles, was centrifuged at 10,000×g for 15min, and the supernatant was used as crude enzyme solution from rumen fluid (RFE, rumen fluid enzymes). The microbial pellet formed was resuspended in 500 mL of sodium phosphate buffer, and cells were disrupted by ultrasonication under anaerobic conditions for 2 min with 3 second intervals on ice using the Vibra Cell^{TM} sonicator (Sonics and Materials Inc., Danbury, Connecticut, USA) set at maximum output. The sonicate was then centrifuged (10,000×g for 15 min) and the supernatant containing the released soluble proteins was used as crude enzyme solution from cell bound enzymes (CBE).

Determination of enzyme activities

Enzyme activity assays were performed in triplicate. The crude enzyme solutions fractionated from the rumen contents were assayed with the various kinds of substrates and buffers under the conditions listed in Table 1. After each assay as shown in Table 1, activity was terminated by denaturing the enzyme by placing the reaction tubes in boiling water for 10 min. After cooling on ice, each sample was centrifuged (14,000×g for 3 min) (Lever, 1977), and liberated reducing sugars in the supernatant was analyzed spectrophotometrically by the method of Nelson-Somogi (Nelson, 1944; Somogi, 1952). One international unit (IU) of each enzyme activity was defined as the amount of enzyme that released one μmol of reducing sugar (expressed as glucose, xylose or arabinose equivalent) per min per mL of crude enzyme solution.

Activity on artificial substrates was determined by monitoring the release of ρ-nitrophenol (ρNP). Reactions were stopped by the addition of 1 mL of 1 M Na_2CO_3. One IU of each enzyme activity was defined as micromoles of ρNP released from ρNP-β-D-glucopyranoside, ρNP-β-D-xylopyranoside, ρNP-β-D-cellobioside, ρNP-α-L-arabinofuranoside, ρNP-β-D-acetate and ρNP-β-D-glucopyranoside per min per mL of crude enzymes.

Pectate and pectin lyase activities were measured by recording the change in absorbance at 232 nm during incubation of enzyme solutions with polygalacturonic acid and pectin (Tagawa and Kaji, 1988). Polygalacturonase activity was estimated according to Tagawa and Kaji (1988) via a reducing sugars determination. Crude enzyme solution (0.5 mL) was incubated with 0.5 mL of 0.5% polygalacturonic acid and 1 mL of 100 mM phosphate buffer (pH 6.0) containing 6 mM EDTA for 60 min at 39°C. Reactions were stopped by immersing tubes in a boiling water bath for 5 min. After cooling and centrifugation, reducing sugars were estimated in supernatant fluid (Lever, 1977).

As protease is one of most important hydrolytic enzymes in the rumen, total proteolytic activity was measured by the hyrolysis of azocasein at 39°C. The reaction mixture consisted of 900 μl of substrate solution (0.1% azocasein in 0.1 M sodium phosphate buffer, pH 7.5) and 100 μl of enzyme sample. The mixture was incubated for 30 min at 39°C, and the reaction was stopped by placing the samples on ice and adding 500 μl of a 15% trichloroacetic acid solution. The sample was then spun in an Eppendorf microcentrifuge (12,000 rpm for 3 min) to remove any precipitate. The absorbance at 440 nm of the

Table 1. The experimental conditions for assays of cellulolytic, hemicellulolytic, pectinolytic and other enzyme activities.

Enzymes	EC number	Substrates	Reaction		Buffer[2]	End product [3]
			Temp	Time[1]		
Cellulolytic enzymes						
β-D-endoglucanase	EC 3.2.1.4	CMC[4], sodium salt	39°C	60	SP	Glucose
β-D-exoglucanase	EC 3.2.1.91	Avicel, medium viscosity	39°C	120	SP	Cellobiose
β-D-glucosidase	EC 3.2.1.21	ρNP-β-*D*-glycopyranoside	50°C	30	CP	ρNP [5]
β-D-fucoside fucohydrolase	EC 3.2.1.38	ρNP-β-*D*-fucopyranoside	50°C	30	CP	ρNP
Xylanolytic enzymes						
β-D-xylanase	EC 3.2.1.8	Oat spelt xylan	39°C	60	SP	Xylose
β-D-xylosidase	EC 3.2.1.37	ρNP-β-*D*-xylopyranoside	50°C	30	CP	ρNP
Acetyl esterase	EC 3.1.1.6	ρNP-acetate	50°C	30	CP	ρNP
α-L-arabinofuranosidase	EC 3.2.1.55	ρNP-α-*L*-arabinofuranoside	50°C	30	CP	ρNP
Pectinolytic enzymes						
Polygalacturonase	EC 3.2.1.15	Polygalacturonic acid	39°C	60	SP	GA[6]
Pectate lyase	EC 4.2.2.2	Polygalacturonic acid	39°C	60	SP	GA
Pectin lyase	EC 4.2.2.10	Pectin	39°C	60	SP	GA
Other enzymes						
β-amylase	EC 3.2.1.2	Water soluble starch	39°C	60	SP	Glucose
Endo-arabinase	EC 3.2.1.99	Apple pomace	39°C	60	SP	Glucose
β-D-gluanase (Laminarinase)	EC 3.2.1.39	Laminarin	39°C	60	SP	Glucose
β-D-glucanase (Lichenase)	EC 3.2.1.73	Lichenan	39°C	60	SP	Glucose
β-D-glucanase (Pechimanase)	EC 3.2.1.	Pechiman	39°C	60	SP	Glucose
Protease	EC 3.4.24.4	Azocasein	39°C	30	SP	Azopeptide

[1] Reaction times were expressed as min.
[2] SP, 0.50M sodium phosphate buffer (pH 6.8) except for analysis of protease; CP, 0.10M citric phosphate buffer (pH 6.8).
[3] Reducing sugars (polysaccharide-degrading enzymes) or ρ-nitrophenol (ρNP; glycoside-degrading enzymes) released by 1 mL of crude enzymes in min.
[4] CMC, Carboxymethylcellulose.
[5] ρNP, para-nitrophenol.
[6] GA, Galacturonic acid.

supernatant was measured using water to zero the spectrophotometer. One unit of protease activity is defined as the micrograms of azocasein hydrolyzed hr^{-1} mL crude $enzyme^{-1}$.

Total protein concentrations were determined using the Bio-Rad (Bio-Rad Laboratories, Richmond, California, U.S.A.) protein determination reagent with bovine gamma-globulin as standard. Specific activity is expressed as μmol reducing sugar (polysaccharidases) or μ-nitrophenol (glycosidases) released per mg protein per min. Chemicals including laminarine, lichenan and pechiman were purchased from MegaZyme (North Rocks, Sydney, NSW, 2151, Australia). The other chemicals used in the enzyme assays were purchased from Sigma (Sigma Chemical Co., St. Louis, M.O., USA).

Statistical analysis

Statistical differences were determined by an analysis of variance with mean separations performed by Duncan's new multiple range test using general linear model procedures of SAS (SAS Inst., Inc., Cary, N.C., USA), and P values of <0.01 were considered significant.

RESULTS AND DISCUSSION

Activities of cellulolytic enzymes

The activities of four cellulolytic enzymes in whole rumen contents, rumen fluid, feed associated and cell bound fractions from rumen digesta of Hereford bulls fed 100% alfalfa hay diets were determined (Table 2). The total and specific enzymatic activities varied considerably in the fractions. The activity of β-1,4-D-endoglucanase, which attacks carboxymethylcellulose or phosphoric acid-swollen cellulose in a random fashion, was the highest amongst the cellulolytic enzymes and mainly located in the CBE fraction ($p<0.01$). The activity of β-1,4-D-exoglucanase, which degrades cellulose by splitting off cellobiose units from the non-reducing end of the chain, was highest in WRE fraction but lowest in CBE fraction. Also, the activity of β-1,4-D-endoglucanase showed highest activity in WRE fraction.

It was found that polysaccharide-degrading enzyme such as β-1,4-D-endoglucanase was located mainly in the CBE fractions and glycoside-degrading enzymes such as β-D-glucosidase and β-D-fucosidase were located in the rumen fluids ($p<0.01$). It was interesting that β-D-glucosidase activity was mainly located in the RFE fractions in the present study, because β-D-glucosidase is known to be an intracellular enzyme in most microorganisms (Yazdi et al., 1990). Cellobiose is soluble and potentially transportable through the membrane, and therefore does not require extracellular enzymes for its utilization. However, it is possible that the β-D-glucosidase and β-D-fucosidase activity detected from the rumen fluids

Table 2. Cellulolytic enzyme activities in whole rumen contents, rumen fluid, feed associated and cell bound fractions from rumen digesta of Hereford bulls fed 100% alfalfa diets

Enzymes	Enzyme activity distributed in			
	WRE[1]	RFE[2]	FAE[3]	CBE[4]
Total enzyme activity (IU)[5]				
β-1,4-D-endoglucanase	1183.6±20.39[a*]	362.7±12.80[c]	424.0±17.03[c]	596.3±11.26[b]
β-1,4-D-exoglucanase	38.6±13.33	18.2±1.38	16.6±3.00	8.0±0.28
β-glucosidase	27.7±0.66[a]	17.0±0.66[b]	12.1±0.17[c]	6.8±0.26[d]
β-fucosidase	33.0±0.72[a]	26.5±0.55[b]	18.9±0.22 [c]	7.5±0.15[d]
Specific activity (IU·mg protein^{-1})[6]				
β-1,4-D-endoglucanase	720.2±19.43[a]	206.7± 9.03[c]	379.0±27.93[b]	761.9±70.04[a]
β-1,4-D-exoglucanase	22.5±6.78	10.4±0.99	14.6±2.43	10.2±0.62
β-glucosidase	16.9±0.37[a]	9.7±0.66[b]	10.8±0.55[b]	8.7±0.90[b]
β-fucosidase	20.2±0.10[a]	15.1±0.69[b]	16.9±0.84[ab]	9.5±0.83[c]

[1] WRE (Enzymes located in whole rumen contents); rumen contents including both of liquid and solid fractions were homogenized and centrifuged, and the supernatant was assayed.
[2] RFE (Enzymes located in rumen fluids); rumen contents were centrifuged and the supernatant was assayed.
[3] FAE (Enymes associated with feed particles); feed particles in rumen contents were separated manually, washed with buffer, resuspended in an equal volume of buffer, homogenized, centrifuged and supernatant was assayed.
[4] CBE (Enzymes bound with microbial cells); rumen microbial cell fraction was separated by centrifugation, suspended in an equal volume of buffer, sonicated, centrifuged, and the supernatant was assayed.
[5] IU, Enzyme activities are expressed as μmol reducing sugars (polysaccharide-degrading enzymes) or ρ-nitrophenol (ρNP; glycoside-degrading enzymes) released by 1 mL of crude enzymes in min.
[6] IU·mg protein^{-1}, specific activities (μmol reducing sugars or ρNP released mg^{-1} protein min^{-1}.
* Each value represents Mean±standard error. In the same row Mean with different superscript letters are significantly different ($p<0.01$).

could be caused by the lysis of rumen microorganisms, base on reports by Wells and Russell (1996) who indicated that the ratio of dead (lysed) to live cells in ruminal fluids may be as great as 10:1. Gong and Tsao (1979) also reported that β-D-glucosidase was released only by autolysis of cells.

The β-1,4-D-endoglucanase specific activity in the FAE fraction constituted a larger proportion (28.2%) than that in the RFE fraction (15.3%). These results are similar to the results of Martin and Michalet-Doreau (1995) who reported that the amounts of ^{15}N-labelled microbial biomass in the different fractions of ruminal contents showed that the solid-adherent microorganisms constituted a large proportion (74%) of the total rumen microbial mass. Craig et al. (1987a,b), Forsberg and Lam (1977), and Legacy-Carmier and Bauchart, 1989) have also shown that particle associated cells constituted a large proportion (70-80%) of the total rumen microbial population. These results indicated that adhesion of ruminal microorganisms, especially cellulolytic organisms, to plant cell walls is quite important with regards to the degradation of cellulosic materials.

Activities of hemicellulolytic enzymes

Hemicellulolytic enzyme activities found in rumen compartments are given in Table 3. Although a number of esterases, which are required for degradation of xylan (acetylxylan esterase and feruloyl esterase), were not assayed specifically, it might be assumed that the activities of the various enzymes required for hydrolysis of xylan into monosaccharides have been detected at high levels in this study. It is known that 30-48% of plant cell walls, is a very complex material (Chesson et al., 1986) with xylan normally present as one of the major polymers. As with the cellulolytic enzymes activities, xylan-degrading enzymes were mainly located in the cell bound fraction. However, glycoside-degrading enzymes such as acetylesterase and arabinofuranosidase (but not xylosidase activity) were located in the rumen fluid fraction.

The specific xylanase activity of FAE two times as high as that of RFE. This is in agreement with the results of Martin and Michalet-Doreau (1995) as discussed above. However, although it is not directly to compare pure culture results, it seems different from the above results obtained in pure culture considering that more than 80% of xylanase was released to the cell-free fluid of a *Fibrobacter succinogenes* culture grown with cellulose (Forsberg et al., 1981).

Even though the nature and composition of diet, the host animal, the feeding time and the sampling time after feeding can affect enzyme activities in the rumen, our results indicate that the specific activities of the enzymes tested predominantly are associated with the CBE fraction. The actual contribution of this high activity to overall feed digestion was not studied in this experiment, yet it may exert an import role in plant cell wall degradation in the rumen.

Activities of pectinolytic and other enzymes

The activities of three pectinolytic enzymes in whole rumen contents, rumen fluid, feed associated and cell bound

Table 3. Hemicellulolytic enzyme activities in whole rumen contents, rumen fluid, feed associated and cell bound fractions from rumen digesta of Hereford bulls fed 100% alfalfa diets

Enzymes	Enzyme activity distributed in			
	WRE[1]	RFE[2]	FAE[3]	CBE[4]
Total enzyme activity (IU)[5]				
Xylanase	1,751.3±26.53[b*]	528.6±29.03[d]	765.8±28.42[c]	1,572.6±22.68[a]
β-xylosidase	55.2±0.93[a]	37.5±2.12[b]	22.9±0.37[c]	22.5±0.17[c]
Acetylesterase	345.8±5.05[b]	431.1±2.64[a]	179.6±2.19[c]	74.5±1.45[d]
α-L-arabinofuranosidase	39.2±0.29[a]	37.6±0.38[b]	21.5±0.39[c]	10.4±0.20[d]
Specific activity (IU·mg protein^{-1})[6]				
Xylanase	1,068.6±53.48[b]	300.2±11.34[d]	682.9±40.17[c]	1,995.5±125.16[a]
β-xylosidase	33.7±1.77[a]	21.5±1.62[b]	20.4±1.00[b]	28.6±1.94[a]
Acetylesterase	210.9±10.01[a]	245.9±7.74[a]	160.4±9.03[b]	95.1±8.59[c]
α-L-arabinofuranosidase	23.9±0.97[a]	21.4±1.43[ab]	19.2±1.01[b]	13.2±1.12[c]

[1,2,3,4,5 and 6] As Table 2.

fractions from rumen digesta of Hereford bulls fed alfalfa hay diets were determined (Table 4). The activity of pectate lyase was the highest, while polygalacturonase activity was the lowest among pectinolytic enzymes tested in the present study. The activities of pectinolytic enzymes were located mainly in RFE fraction. Specific pectinase activity was located mainly in the microbial cell as with the cellulolytic and hemicellulolytic enzyme activities. However the pectinase specific activity of the FAE fraction was lower than that of RFE fraction in contrast to cellulolytic and hemicellulolytic enzymes activities. On the other hand, pectate lyase activities were higher in the rumen fluid fraction (more than 50%) than in the other fractions, and only little difference was found between FAE fraction and CBE fraction.

Enzymes in the rumen are diverse, and include plant cell wall polymer-degrading enzymes (e.g. cellulolytic, hemicellulolytic and pectinolytic enzymes), amylase, proteases, phytases and specific plant toxin-degrading enzymes. The variety of enzymes present in the rumen arises not only from the diversity of rumen microbial community but also from the multiplicity of fibrolytic enzymes produced by individual microorganisms (Doerner and White, 1990; Malburg and Forsberg, 1993; Flint et al., 1994; Ali et al., 1995). The efficient digestion of complex substrates in the rumen requires the coordinated activities of many enzymes. Many of the bacterial, protozoan and fungal inhabitants of the rumen exhibit proteolytic and amylolytic activities (Coleman, 1986; Wallace and Cotta, 1988; Michel et al., 1993; Attwood and Reilly, 1995). In our experiments, proteolytic and amylolytic activities in the rumen were also detected at high levels. The relative distributions of specific enzymatic activities varied considerably between the WRE, RFE, FAE and CBE fractions ($p<0.01$). The distribution of specific protease activity was 37.7, 22.1 and 40.2%, and of amylase activity was 51.6, 18.2 and 30.2% for the RFE, FAE and CBE fractions, respectively. These results indicated that protease is located mainly in the rumen fluid and with the cell, whereas amylase was located mainly in rumen fluid. Arabinase, laminarinase and lichenase activities were detected in all fractions while pechimanase activity was detected neither from the FAE fraction nor from the CBE fraction , but in the RFE fraction.

In summary, it was found that polysaccharide-degrading proteins such as β-1,4-D-endoglucanase, β-1,4-D-exoglucanase, xylanase and pectinase enzymes were located mainly in the cell bound (CBE) fraction. However, glycoside-degrading enzymes such as β-D-glucosidase, β-D-fucosidase, acetylesterase, and α-L-arabinofuranosidase were located in the rumen fluids fraction. Protease was

Table 4. Pectinolytic enzyme activities in whole rumen contents, rumen fluid, feed associated and cell bound fractions from rumen digesta of Hereford bulls fed 100% alfalfa diets

Enzymes	Enzyme activity distributed in			
	WRE[1]	RFE[2]	FAE[3]	CBE[4]
Total enzyme activity (IU)[5]				
Polygalacturonase	195.5±4.38[a]*	204.2±18.34[a]	70.8±1.32[b]	83.8±2.07[b]
Pectate lyase	440.4±14.49[a]	431.8±10.88[a]	170.4±5.62[c]	244.5±5.36[b]
Pectin lyase	190.0±17.86[a]	223.0±24.72[a]	76.5±7.37[b]	48.6±19.23[b]
Specific activity (IU·mg protein^{-1})[6]				
Polygalacturonase	119.4±7.01[a]	117.2±13.58[a]	63.1±2.77[b]	106.4±7.49[a]
Pectate lyase	267.7±7.60[a]	245.7±1.41[a]	152.6±11.73[b]	312.5±29.04[a]
Pectin lyase	116.8±15.28[ab]	127.9±16.19[a]	67.6±4.53[b]	56.7±18.01[b]

[1,2,3,4,5 and 6] As Table 2.

Table 5. Other enzyme activities in whole rumen contents, rumen fluid, feed associated and cell bound fractions from rumen digesta of Hereford bulls fed 100% alfalfa diets

Enzymes	Enzyme activity distributed in			
	WRE[1]	RFE[2]	FAE[3]	CBE[4]
Total enzyme activity (IU)[5]				
Protease	125.6±3.83[a*]	84.8±2.52[b]	31.9±1.44[c]	40.1±1.59[c]
Amylase	637.9±14.80[a]	439.0±16.53[b]	98.6±2.44[c]	115.4±1.23[c]
β-1,3-D-glucanase	269.8±9.65[a]	99.8±0.68[c]	126.7±4.32[b]	128.6±5.66[b]
β-1,3-1,4-D-glucanase	534.9±11.79[a]	324.0±9.49[b]	206.0±4.90[d]	250.5±7.35[c]
Arabinase	1548.1±29.19[a]	1582.7±24.28[a]	724.3±22.80[b]	757.3±21.52[b]
Pechimanase	32.7±10.78	nd	nd	nd
Specific activity (IU·mg protein^{-1})[6]				
Protease	76.7±4.70[a]	48.3±1.85[b]	28.3±0.84[c]	51.4±5.72[b]
Amylase	390.2±25.68[a]	250.9±14.82[b]	88.2±5.80[c]	146.9±11.45[c]
β-1,3-D-glucanase	164.1±5.71[a]	56.9±1.74[c]	113.2±7.14[b]	162.2±6.78[a]
β-1,3-1,4-D-glucanase	326.8±19.13[a]	184.3±0.24[b]	184.0±11.01[b]	319.3±26.97[a]
Arabinase	946.4±57.41[a]	901.4±14.55[a]	648.5±48.79[b]	961.0±67.11[a]
Pechimanase	20.7±7.11	nd	nd	nd

[1,2,3,4,5 and 6] As Table 2.

located mainly in rumen fluid and microbial cells, whereas amylase was located mainly in the rumen fluid.

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