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Original article

Effect of extraction method on activities of polysaccharide-depolymerase enzymes in the microbial population from the solid phase in the rumen

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Summary — Samples of rumen digesta were collected from 2 cows fed with a 100:0 or 40:60 forage/concentrate diet 1 h before and 2 h after feeding. Enzymes from particle-associated microorganisms were extracted by 5 different methods, including physical (freezing, grinding and sonication) or chemical and enzymatic (autolysis in a buffer, lysozyme and CCl_4) treatments. Hydrolytic enzyme activities were measured on 3 polysaccharidic substrates: xylan, carboxymethylcellulose and Avicel cellulose. Contamination of enzyme preparations by feed proteins was evaluated. For the 3 substrates, the effect of feeding (sampling time and diet) on enzyme activities was independent of extraction method. Maximal activities were obtained with treatments including lysozyme and CCl_4 or grinding in liquid nitrogen. Contamination of enzyme preparations by feed proteins was higher with chemical and enzymatic methods than with physical treatments.

rumen / particle-associated microorganism / enzyme activity / polysaccharidedepolymerase

Résumé — Effet de la méthode d'extraction sur l'activité des polysaccharidases de la population microbienne de la phase solide dans le rumen. *Des échantillons de contenu ruminal ont été prélevés 1 h avant et 2 h après le repas, sur 2 vaches recevant soit une ration à base de foin, soit une ration à base de foin et d'orge dans les proportions 40:60. Les enzymes de la population microbienne adhérente à la phase solide ont été extraites par 5 méthodes différentes faisant intervenir des traitements physiques (congélation, broyage, sonication) ou chimiques et enzymatiques (autolyse dans un tampon, lysozyme et CCl_4). L'activité hydrolytique des enzymes a été mesurée sur 3 substrats polysaccharidiques : le xylane, la carboxyméthylcellulose et la cellulose Avicel. La contamination des préparations enzymatiques par des protéines alimentaires a été évaluée. Quel que soit le substrat considéré, l'effet de l'alimentation (heure de prélèvement, ration) sur les activités a été le même pour les 5 traitements. Les activités enzymatiques les plus importantes ont été obtenues avec les traitements faisant intervenir le lysozyme et le CCl_4 ou un broyage dans l'azote liquide. La contamination des préparations enzymatiques par des protéines alimentaires a été plus importante avec les traitements chimiques et enzymatiques qu'avec les traitements physiques.*

rumen / microorganisme adhérent / activité enzymatique / polysaccharide-dépolymérase

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INTRODUCTION

Digestion of plant cell walls in the rumen depends on the microbial ecosystem and its activity. Microorganisms that utilise plant structural polysaccharides as their major energy source have to be closely associated with plant particles entering the rumen (Akin, 1979; Chesson and Forsberg, 1988). The microbial population adhering to feed particles is predominant in the rumen (Merry and McAllan, 1983) and is directly responsible for their degradation (Silva *et al*, 1987). The ability of cell-wall degraders to adhere to plant material is of primary importance and appears an essential first stage in the digestive process (Latham *et al*, 1979). Adherent fibre-degrading bacteria can help put cell-bound enzymes into contact with the substrate, thereby ensuring effective digestion of cellulosic components (Chesson and Forsberg, 1988). The measurement of particle-bound microbial enzyme activities can be used to predict the rate and extent of fibre degradation in the rumen (Silva *et al*, 1987). This measurement requires the extraction of these enzymes from microbes adhering to rumen content. Extractions by sonication (Williams and Strachan, 1984; Martin *et al*, 1993) or lysozyme and carbon tetrachloride (CCl_4) treatment (Silva *et al*, 1987; Huhtanen and Khalili, 1992; Bowman and Firkins, 1993) are the most commonly used. However, since this extraction is not complete, the validity of the methods is questionable.

The aim of this experiment was to compare 5 extraction methods, including physical (freezing, grinding, and sonication) and chemical and enzymatic (autolysis in a buffer, lysozyme and CCl_4) treatments, by studying both extraction yield and variation in response to dietary factors. Extraction yield is of particular importance for measurements of enzymatic activity on crystalline substrates like Avicel cellulose. Hydrolysis of this crystalline substrate requires the activity of a complex cellulasic system, including both exocellobiohydrolases and endo-1,4-β-D-glucanases, whereas hydrolysis of other more soluble substrates, such as carboxymethylcellulose (CMC), depends mainly on the activity of endo-1,4-β-D-glucanases. The use of pure Avicel cellulose as a substrate thus provides a situation as close as possible to plant-cell-wall cellulose breakdown (Wood and Bhat, 1988). The quantity of enzyme extracted determines the precision of measurements and their relative activities. We have compared, therefore, different extraction methods by studying variations in enzymatic activities with length of time after feeding (Williams *et al*, 1989) and dietary composition (Huhtanen and Khalili, 1992).

MATERIALS AND METHODS

Animals and feeding

Two dry Jersey cows, fitted with ruminal cannula, were fed (7 kg DM/animal/d) with a forage/concentrate diet consisting of hay, straw and barley at ratios of 90:10:0 (cow H) and 30:10:60 (cow HB). The animals were fed twice a day at 08.00 and 17.00 h. They also received a mineral/vitamin block and were given free access to water. An adaptation period of 6 weeks was allowed before the measurements were made.

Isolation of solid-associated microbes and enzyme preparation

Three representative samples of rumen digesta (approximately 300 g) were collected *via* the cannula 2 h and 23 h post-feeding, on 3 consecutive days. The samples were collected in a capped bottle to avoid contact with air and immediately transported to the laboratory. They were strained through a 100-μm nylon filter, under a CO_2 stream, to separate the solid phase from which the solid-associated microbial enzymes were isolated under anaerobic conditions. The solid-phase

samples were gently agitated in a bottle with anaerobic salt solution (Coleman, 1978) prewarmed at 39°C (1 g fresh weight of solids in 4 ml diluent) to remove the non-adherent population associated with particles and were then recovered by filtration (100 μm). The surface of the filter was gassed with CO_2. The washed digesta solids that contained the adherent population were lacerated under anaerobic conditions and submitted to the 5 following treatments, under the same conditions as described previously by the authors referenced.

In *Treatment S*, 5 g (fresh weight) of the solid was suspended in 25 ml pre-cooled (4°C) anaerobic (0.025 mol/l 2-(*N*-morpholino) ethane sulphonic acid (MES)) buffer (pH 6.5) containing 1 mmol/l dithiothreitol (DTT). The microorganisms present in the suspension were disrupted by ultrasonic disintegration with a Labsonic U (B Braun) at 4°C under anaerobic conditions. Four 30-s periods of sonication with 30-s intervals were used (Williams and Strachan, 1984). *Treatment GS* was similar to *Treatment S*, but washed digesta solids were ground in liquid nitrogen and the suspension in MES DTT buffer was frozen and thawed before sonication. *Treatment G* was similar to *Treatment GS*, except that sonication was not carried out.

In Treatment L^+ and *Treatment* L^- 2.5 g (fresh weight) of the solid was incubated under anaerobic conditions at 39°C for 3 h in 25 ml MES DTT buffer with (L^+, Huhtanen and Khalili, 1992) or without (L^-) 1.25 ml lysozyme (50 mg/ml) and 3.215 ml CCl_4.

After treatment, the microbial population was recovered by centrifugation in the same conditions as described previously by the authors referenced: 15 000 *g* (*Treatments S, GS, G*) or 26 000 *g* (*Treatments* L^+, L^-) for 15 min at 4°C. The supernatant fraction was stored under a CO_2 headspace in capped tubes at −80°C until used for enzyme assays. For all treatments, the dry matter (DM) content of samples before suspension in the buffer was determined.

Enzyme assay procedures

Hydrolysis of xylan (Sigma no X 0502), sodium carboxymethylcellulose (CMC, medium viscosity, Sigma no C 5678) and Avicel (Macherey Nagel, no 81629) were assayed by measuring the amount of reducing sugars formed after incubation at 39°C for 60 min. The reaction was stopped by heating at 100°C for 5 min. The reducing sugars (RS) released were quantified by the *p*-hydroxybenzoic acid hydrazide method (Lever, 1977) with glucose or xylose as the standard. Xylan (2 mg/ml), CMC (2 mg/ml) and Avicel (10 mg/ml) were prepared in MES DTT buffer. Incubation mixtures consisted of either 0.1 ml of enzyme preparation and 1 ml of substrate (CMC and xylan assays) or 0.5 ml of enzyme preparation and 1.5 ml of substrate (Avicel assay). Enzyme and substrate controls were also performed simultaneously by replacing substrate and enzymes by MES DTT buffer, respectively.

The protein content of enzyme preparations was determined by the Pierce method (Pierce and Suelter, 1977) using bovine serum albumin standards. Specific activity was expressed as nmol RS released per mg protein in 1 h, and total activity as μmol RS released per g DM in 1 h.

Estimation of feed proteins in enzyme preparations

Eventual presence of feed proteins in enzyme preparations, which would overestimate the extent of extraction (mg proteins/g DM), would underestimate specific activities (nmol RS released per mg protein in 1 h). In order to estimate this contamination and its influence on the different extraction methods, 12 g of hay and barley in proportions 100:0 and 40:60 were incubated at 39°C in 1 l of artificial saliva buffer (Vérité and Demarquilly, 1978) under stirring for 2 h. After filtration and washing as described previously, the particles retained on the filter were subjected to the same 5 enzyme extraction methods as the ruminal samples, without taking any anaerobic precautions. Protein concentration was determined by the Pierce method.

Statistical analysis

Enzyme activities of rumen contents were analysed as a split-plot design using a GLM procedure (SAS Institute, 1985). The activity obtained for each sample was the experimental unit ($n = 12$ for each treatment). Feeding (*ie* combination of diet and sampling time) was the main plot factor and extraction method was the subplot. The main-

plot error consisted of the replication x feeding interaction. The subplot error ($df = 32$) was used to test the significance of the extraction method effect and of its interaction with feeding main factor. Extraction method differences were separated by Duncan's multiple range test when the F test was significant ($P < 0.05$).

Protein release in enzyme preparations was analysed with a similar model, with incubation site as the main-plot factor and extraction method as the subplot.

RESULTS

Enzyme activities

For the 3 substrates, total (table I) and specific (table II) activities of enzymes extracted by sonication alone *(Treatment S)* were always lower than activities of enzymes extracted by the other methods.

Xylanase total activity (table I) was significantly higher with grinding treatments (*Treatments G* and *GS*) than without grinding *(Treatment S)* (294 *vs* 72 µmol RS/g DM/h), and also after action of lysozyme/CCl_4 (*Treatment* L^+) compared with action of buffer only (*Treatment* L^-) (278 *vs* 171 µmol RS/g DM/h). CMCase total activity was lower after the sonication alone (5.9 µmol RS/g DM/h), and was similar for the other 4 methods (27.7 µmol RS/g DM/h). With Avicel, the highest total activities were obtained with grinding or lysozyme/CCl_4 treatments (21.1 and 22.1 µmol RS/g DM/h, respectively). When samples were incubated in the buffer alone, 75% of the maximal avicelase activity was extracted.

Xylanase specific activity (table II) was higher with grinding treatments than with the sonication alone (8 670 *vs* 3 690 nmol RS/mg protein/h), and with lysozyme/CCl_4 (7 320 nmol RS/mg protein/h) than without (5 760 nmol RS/mg protein/h). CMCase specific activity obtained with the sonication alone (300 nmol RS/mg protein/h) was lower than with the other 4 methods (845 nmol RS/mg protein/h). A similar trend was observed for avicelase (342 *vs* 622 nmol RS/mg protein/h).

The mean intra-diet and sampling time variability was the same whatever the extraction method used. The calculated mean variances were 45, 4.1 and 2.8 for xylanase, CMCase and avicelase total activities, respectively, and 1524, 140 and 89 for xylanase, CMCase and avicelase specific activities, respectively.

Table I. Effect of extraction method on total activities of polysaccharide-polymerase enzymes (µmol reducing sugar/g DM in 1 h) in the particle-bound microbial population.

Substrate	*Extraction method*					*SEM*	*Statistical significance*		
	S	*G*	*GS*	L^+	L^-		*Extraction method*	*Feeding*	*Extraction method x feeding*
Xylan	72 [c]	291 [a]	297 [a]	278 [a]	171 [b]	56	***	***	*
CMC	5.9 [b]	28.6 [a]	27.5 [a]	28.8 [a]	26.0 [a]	4.3	***	***	***
Avicel	6.6 [d]	23.2 [a]	19.0 [bc]	22.1 [ab]	17.3 [c]	4.1	***	***	*

The values are means of 12 determinations. * $P < 0.05$; *** $P < 0.001$. [a,b,c,d] Extraction method significance by F test; means in the same row with different superscripts differ ($P < 0.05$).

Table II. Effect of extraction method on specific activities of polysaccharide-depolymerase enzymes (nmol reducing sugar/mg protein in 1 h) in the particle-bound microbial population.

Substrate	*Extraction method*					*SEM*	*Statistical significance*		
	S	*G*	*GS*	*L+*	*L−*		*Extraction method*	*Feeding*	*Extraction method x feeding*
Xylan	3 690 [d]	9 180 [a]	8 160 [ab]	7 320 [b]	5 760 [c]	1 676	***	***	NS
CMC	300 [c]	897 [a]	771 [b]	801 [ab]	909 [a]	143	***	**	***
Avicel	342 [c]	729 [a]	540 [b]	588 [b]	630 [b]	116	***	**	*

The values are means of 12 determinations. NS: not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
[a,b,c,d] Extraction method significance by F test; means in the same row with different superscripts differ ($P < 0.05$).

Response to feeding

The responses of the enzymatic activities to feeding, resulting from diet and sampling time, were very important (tables I and II). For all extraction methods, total xylanase and avicelase activities were lower in the cow receiving a mixed forage/concentrate diet (192 and 15.2 µmol RS/g DM in 1 h, respectively), compared with the cow fed only forage (251 and 20.0 µmol RS/g DM in 1 h, data not shown). In contrast, the total CMCase activity (23.4 µmol RS/ g DM in 1 h) and the specific activities of the 3 enzymes were identical in the 2 cows (6 820, 736 and 566 nmol RS/mg protein in 1 h for xylanase, CMCase and avicelase, respectively, data not shown). A reduction in both total and specific activities was observed after feeding with the 5 methods in the 2 cows. The average decrease was 48% for xylanase, 56% for CMCase and 22% for avicelase (data not shown).

The amplitude of responses to these feeding factors was variable according to extraction method, especially in the case of CMCase ($P < 0.001$). Thus, the decrease in total CMCase activity after feeding appeared to be non-significant when sonication alone was used (–5.6 units), compared with the other 4 extraction methods (–21.0 units in mean).

Therefore, the amplitude of responses to feeding on enzymatic activities was variable according to extraction method, but the trends were the same.

Release of feed proteins in enzyme preparations

With all the methods, the release of proteins from the rumen contents was, as expected, higher than the release from feed incubated for 2 h in artificial saliva (table III). The amount of feed proteins extracted from samples incubated in saliva was irrespective of the feed and was higher with enzymatic and chemical (22.5 mg/g DM on average) than with physical treatments (11.7 mg/g DM on average). Thus, the proportion of feed proteins in the enzyme preparations from rumen contents would be smaller with these physical treatments, including grinding in liquid nitrogen, with or without sonication.

Table III. Effect of extraction method on release of proteins in the enzyme preparations after a 2-h incubation period (mg proteins released/g DM).

Incubation site	*Extraction method*					*SEM*	*Statistical significance*		
	S	*G*	*GS*	*L+*	*L−*		*Extraction method*	*Incubation site*	*Extraction method x incubation site*
Buffer	10.6 ^{Bb}	11.9 ^{Bb}	12.7 ^{Bb}	23.1^{Ba}	21.8 ^{Ba}	4.5	***	***	NS
Rumen	20.6 ^{Ac}	31.4 ^{Ab}	34.5 ^{Ab}	42.5 ^{Aa}	31.4 ^{Ab}				

The values are means of 2 (buffer) or 6 (rumen) determinations. NS: not significant; *** $P < 0.001$. ^{A,B} Incubation site significance by *F* test; means in the same column with different superscripts differ ($P < 0.05$). $^{a, b, c}$ Extraction method significance by *F* test; means in the same row with different superscripts differ ($P < 0.05$).

DISCUSSION

Extraction of microorganism enzymes bound to particles *via* methods that involve grinding in liquid nitrogen or lysozyme/CCl_4 gave much higher enzymatic activities than sonication alone. This is true for both specific and total activities. Xylanase and CMCase activities extracted in this experiment by sonication (3 690 and 300 nmol RS/mg protein/h, respectively) were similar to those reported by Martin *et al* (1993), obtained under identical feeding, sampling and analytical conditions (3 090 and 310 nmol RS/mg protein/h, respectively).

The large quantity of enzymes extracted by *Treatment GS* appears to result more from grinding and the freezing/defrosting sequence (in comparison with *Treatment S*) than from the sonication which followed (in comparison with *Treatment G*). The grinding improves microbial accessibility while freezing/defrosting improves rupture of microbial cells. The Gram-negative bacteria, which are predominant in the rumen, are considered to be particularly susceptible to this treatment (Hammond, 1992).

Lysozyme and CCl_4 appeared to have a low specific effect on enzyme extraction, since incubation of digesta in the same buffer alone gave more than 50% of the activities obtained in the presence of lysozyme and CCl_4. In addition to the autolysis of cells suspended in buffer (*Treatment* L^-), lysozyme hydrolyzed the β-1,4-glycosidic linkages of peptidoglycanes of bacterial cell walls (*Treatment* L^+). It is probable that Gram-positive bacteria, whose peptidoglycane is not protected by an external membrane, are particularly susceptible to this hydrolysis. Possibly CCl_4 plays a role in disorganization of bacterial cell walls by its solvent properties.

Few studies have been made to compare techniques for extraction of particle-bound microbial polysaccharidases. Silva *et al* (1987) found no difference between the total microbial CMCase activities extracted by lysozyme/CCl_4 and by sonication. The values obtained by those authors were much higher than those reported here, suggesting that the rates of hydrolysis were measured after shorter incubation periods than in the present trial, but this was not indicated.

The specific activity of microbial enzymes is expressed in nmol RS released/mg proteins in 1 h. These proteins present in the enzymatic preparations are of both microbial and dietary origin. Extraction methods may break down both bacterial and plant cells. To estimate this possible contamination of enzymatic preparations by dietary proteins, feed samples identical to those given to the animals were incubated in artificial saliva and then subjected to the same extraction treatments as the digestas. This method overestimates the importance of dietary proteins in enzymatic preparations since only the soluble protein fraction in the buffer is eliminated before extraction. Thus, the results obtained have only a relative value permitting comparison between extraction methods. The quantities of feed proteins extracted were smaller with physical treatments (freezing, grinding and/or sonication) than with enzymatic and chemical treatments (buffer with or without lysozyme/CCl_4). The proportion of dietary proteins in enzyme preparations would appear to be smaller with treatments involving grinding and freezing compared with enzymatic and chemical treatments or sonication only.

Differences in activities of particle-bound microbial enzymes observed between the 2 diets were in agreement with those reported in literature: diminution of total CMCase and xylanase activities due to starch or sugar supplements (Silva *et al*, 1987; Huhtanen and Khalili, 1992); and diminution of activities after feeding (Williams *et al,* 1989; Bowman and Firkins, 1993; Martin *et al,* 1993). In this trial, differences in activities observed between the 2 cows could not be entirely explained by a diet effect, each cow receiving only one diet. However, the 5 extraction methods gave the same responses to feeding. The amplitude of responses were different and, notably were lower when sonication alone was used.

The extraction methods compared in this experiment thus gave the same responses to feeding. Grinding in liquid nitrogen improved enzyme yield, in terms of activity, compared with sonication alone. The enzymatic and chemical treatments extracted identical quantities of enzymes as grinding, but they also liberated more feed proteins, which means that specific enzyme activity is underestimated.

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