Effect of Nylon Bag and Protozoa on In Vitro Corn Starch Disappearance

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ABSTRACT

An in vitro experiment was carried out to study whether the presence of protozoa in nylon bags can explain the underestimation of the in situ degradation of slowly degradable starch. Corn of a high (flint) and a low (dent) vitreousness variety was ground over a 3-mm screen, weighed in nylon bags with a pore size of 37 μ m, and washed in cold water. Samples of washed cornstarch were incubated in 40-mL tubes with faunated and defaunated ruminal fluid. An additional amount of washed corn, in nylon bags, was inserted in each incubation tube. Incubations were carried out for 0, 2, 4, 6, 12, and 24 h, and starch residue in tube and nylon bag was determined. In general, starch disappearance from the nylon bag was less than from the tube, and was less with faunated than defaunated rumen fluid, but corn variety did not affect starch disappearance. When no protozoa were present, the disappearance of starch from the bags was higher after 6 and 12 h incubation compared with presence of protozoa. However, in the tubes, there was no difference in starch disappearance due to presence or absence of protozoa. Estimated lag time was higher in presence (4.6 h) than absence (3.6 h) of protozoa. It was concluded that the effect of presence or absence of protozoa on starch disappearance differs within or outside nylon bags. The reduced disappearance rate of starch inside the nylon bags in the presence of protozoa helps to explain the underestimation of starch degradation based on the in sacco procedure when compared with in vivo data upon incubation of slowly degradable starch sources.

Key words: protozoa, starch, nylon bag method

INTRODUCTION

Starch is generally considered as completely digestible by ruminants (Offner and Sauvant, 2004), but the site of digestion (rumen or postrumen) may vary be-

tween starch sources (Mills et al., 1999). The site of starch digestion determines whether starch serves as an energy source for ruminal microbes or becomes available in the intestines. Fermentation of starch in the rumen gives rise to high proportions of propionic acid (Bannink et al., 2006), which is a glucogenic precursor, but also considerable amounts of acetic and butyric acid are formed, which are lipogenic. Relative to starch fermented in the rumen, starch digested in the small intestine and absorbed as glucose will more efficiently increase glucogenic supply. In particular, early lactating cows have a high demand for glucogenic nutrients, where feeding extra glucogenic nutrients in comparison to lipogenic nutrients improved energy balance and reduced the severity of ketosis and fatty liver (reviewed by van Knegsel et al., 2005). However, evidence that increased small intestinal starch digestion will benefit the cow in terms of milk energy production and energetic efficiency is equivocal (reviewed by Reynolds, 2006).

The site of digestion can be predicted from degradation characteristics of starch in the rumen. Disappearance of starch from nylon bags, incubated in situ in the rumen, is widely used as a method to determine degradation characteristics. Offner and Sauvant (2004) showed, however, that the effective rumen starch degradation predicted from in situ determination appears to underestimate the in vivo degradation of slowly degradable starch sources including corn and sorghum. This was confirmed by the work of Hindle et al. (2005) who found that cornstarch degradation was 53% in situ and 75% in vivo, whereas wheat starch degradation in situ and in vivo was 86 and 90%.

A possible explanation for this underestimation in situ is the relative high proportion of vitreous or hard endosperm of fully matured corn and sorghum grain compared with other grains. The vitreous endosperm differs from floury endosperm because vitreous endosperm is embedded in a dense protein matrix. The protein matrix functions as a barrier against microbial degradation (McAllister et al., 1990). The proteins within the vitreous endosperm consist mainly of α , β , and δ zeins (Philippeau et al., 2000), which are recog-

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nized as slowly degradable, insoluble proteins. Protozoa are more capable to degrade zeins than bacteria (Ushida and Jouany, 1985; Hino and Russell, 1987). Taylor and Allen (2005) suggested, therefore, that protozoa play a major role in degrading vitreous endosperm. Fondevilla and Dehority (2001) showed that in vitro cornstarch degradation in presence of *Entodinium exiguum* and bacteria in coculture occurred at a faster rate compared with bacteria only. However, in vivo studies with sheep or cattle gave variable results, indicating a decline (Veira and Ivan, 1983) or increase (Mendoza et al., 1993) of ruminal starch degradation upon defaunation. Especially larger protozoa are important in the degradation of insoluble proteins (Williams and Coleman, 1997). Increasing nylon bag pore size from 50 to 100 μm increased the degradation of insoluble soybean protein with protozoa compared with bacteria only (Ushida and Jouany, 1985). This implies that protozoal degradation of insoluble protein can be influenced by the nylon bag pore size. Lindberg and Kaspersson (1984) found a decrease in protozoal count when nylon bag pore size decreased from 36 to 10 μm . Franzolin et al. (2002) did not observe differences in protozoa concentrations between nylon bag pore size of 52 and 100 μm , but observed a 2.3- to 2.6-fold lower protozoa count in the nylon bag content compared with the rumen content of the same cows. Thus, the effect of protozoa on starch degradation may depend on vitreousness and on place (inside or outside nylon bags) of degradation.

The objective of the present experiment was to examine the hypothesis that the in situ method underestimates rumen starch degradation, especially of starch sources with a high proportion of vitreous endosperm, which are slowly degradable. This experiment also examined the hypothesis that this underestimation is due to a lower number of protozoa in nylon bags than in rumen fluid. In an in vitro experiment, we therefore studied the disappearance of 2 cornstarch sources (high and low vitreous endosperm) inside and outside nylon bags and in presence or absence of protozoa.

MATERIALS AND METHODS

Experimental Design

Two corn varieties were used: a flint type, high vitreous endosperm corn (HVC), and a dent type, low vitreous endosperm corn (LVC). The specific density of the kernels, as an indirect indication of vitreousness, was measured as described by Correa et al. (2002). Vitreousness of the kernels was determined as described by Dombrink-Kurtzman and Bietz (1993). The LVC variety had 68.1% vitreous endosperm, whereas the HVC variety had 80.9% vitreous endosperm. Apparent density was 1.166 and 1.217 g/cm^3 and starch content was

60.4 and 59.4% of complete kernels, for LVC and HVC, respectively.

Samples were incubated in vitro suspended in the rumen fluid in the tube and within nylon bags in the same tube. Before incubation, kernels were ground by hammer milling at 1,500 rpm and with a 3-mm sieve. To prevent exchange of starch between tube and bag, only the nonwashout fraction of starch was used. The nonwashout fraction of ground, unprocessed corn has a starch content similar to that of the washout fraction, and the starch content of the water-soluble part of the washout fraction is very small (some 2% of that in the total washout fraction; Yang et al., 2005). Therefore, after grinding corn was inserted in nylon bags with a pore size of 37 μm , and washed in tap water according to the nylon bag procedure used in the Netherlands (Tas et al., 2006). After washing, bags were dried for one hour at 70°C and contents of the bags used for incubation.

For the in vitro incubation, mixed rumen fluid of 2 multiparous midlactating cows was used as inoculum. Both cows were equipped with a large rumen cannula (Bar-Diamond, Parma, ID) with surgery approved by the Animal Experiment Ethical Committee of the Animal Sciences Group, Lelystad, the Netherlands. The cows received a 15% starch TMR with on a DM basis 43.5% grass silage, 15.5% beetpulp, 9.5% soybean meal, 30.0% ground corn, and 1.5% macrominerals and trace elements. Daily DM intake was 18 kg. Feeding time was 0600 h and rumen fluid was collected at 0830 h.

The in vitro incubation was carried out with faunated rumen fluid (FF) and with defaunated rumen fluid (DF). The rumen fluid was continuously flushed with CO_2 to ensure anaerobic conditions and kept at 39°C. The rumen fluid was sieved through a 400- μm sieve and centrifuged at $1,000 \times g$ for 10 min (Beckman J2-21, Beckman Coulter, Fullerton, CA). For the FF, after centrifuging the pellet was mixed again with the supernatant by stirring. For the DF, the supernatant was removed temporarily. Defaunation in the remaining pellet occurred by freezing on dry ice, thawing on warm water, and sonification. After sonification, the pellet was added to the supernatant and stirred. Rumen fluid was mixed (1:2, vol/vol) with an anaerobic buffer/mineral solution as described by Cone et al. (1996).

Two corn varieties, 2 inoculants and 2 locations (in and outside the nylon bag) were distinguished resulting in 8 unique combinations and were incubated for 0, 2, 4, 6, 12, and 24 h. In total 48 tubes (50 mL; BD Falcon Conical Centrifuge Tubes, BD Biosciences, San Jose, CA) were filled with 40 mL of DF or FF mixed with medium, 160 mg of ground, washed-out HVC or LVC, and a nylon bag with 160 mg of ground, washed out HVC or LVC. During the incubation, the tubes were

kept under dark conditions at 39°C and shaken continuously. Incubation was stopped by freezing with dry ice. Tubes were stored at -18°C until analyses.

Starch Analysis

Starch analysis was performed on the ground, washed out corn, the original ground corn and (after thawing) on the complete contents of the tube and of the nylon bag according to ISO-15914. For starch analysis in bags and tubes, no preextraction was applied. Bags were removed from the tube. Tubes were centrifuged at 800 × *g*, the supernatant was removed, and the pellet was placed with 40 mL of demi water into a 100-mL flask. The nylon bags were dried for 3 h at 70°C. After drying, the contents were placed with 40 mL of demi water into a 100-mL flask. The flasks were shaken for 5 min in a boiling water bath. Autoclaving was performed for 3 h at 130°C. After autoclaving, hydrolysis occurred by adding 2.5 mL of 2 *M* acetate buffer pH 4.8, and 0.75 mL of amyloglucosidase solution (80 U/mL), incubating at 60°C for 16 to 18 h. After cooling down in ice water, filtration occurred by adding 2 mL of a potassium hexacyanoferrate (II) solution (0.25 mol/L) and adding 2 mL of zinc acetate (1 mol/L) in acetic acid (0.5 mol/L), and filtrated through filter paper (mn 615¼ Macherey-Nagel GmbH, Düren, Germany). Glucose in the filtrate was analyzed enzymatically by addition of a reagent solution containing 100 mg of NADP, 500 mg of ATP, 2 mL of hexokinase (340 U/mL)/glucose-6-phosphate dehydrogenase (170 U/mL), and a triethanolamine buffer (triethanolamine 0.75 mol/L; Mg²⁺ 0.10 mol/L) adjusted to pH 7.6 with sodium hydroxide (5 *M*). Ten minutes after the addition of the reagent solution, NADPH was measured by means of its absorbance at 340 nm. The NADPH formed is directly proportional to glucose concentration. Starch was calculated by multiplying the amount of glucose by 0.9.

Statistical Analysis

Statistical analyses were carried out with SAS (Version 9.1, SAS Inst. Inc., Cary, NC). The starch residues, expressed as a fraction of the amount present at start of incubation (time 0 h), were analyzed using the GLM procedure with corn source (HVC or LVC), inoculum (FF or DF), incubation type (tube or bag), and incubation time (2, 4, 6, 12, and 24 h) as main factors and all 2- and 3-way interactions included. The starch residues were fitted to a nonlinear model (SAS NLIN procedure) per combination of corn source, inoculum, and incubation type. The model used was

$$R_t = R_0 \quad (t < \text{lag})$$

$$R_t = R_0 e^{-[k_d (t - \text{lag})]} \quad (t \geq \text{lag}),$$

where R_t is the residue at time t (h) expressed as a fraction of the incubated amount (R_0), k_d (%/h) is the fractional rate of degradation, and lag (h) is the time lag before fermentation commences.

Parameters k_d and lag were analyzed using the GLM procedure with corn source, inoculum and incubation type as main factors and 2-way interactions included. The Tukey test was used to test for all pairwise comparisons among means when the F -value of main effects or of interactions was significant ($P < 0.05$).

RESULTS

At zero incubation time, tubes contained on average 5.3 mg more starch than the amount of cornstarch weighed into the tube, and nylon bags contained on average 0.4 mg more starch than the amount of cornstarch weighed into the nylon bags. The extra starch was included in the initial amount of starch. This additional starch indicates that dietary or microbial starch was present in the fluid before the incubation started.

Starch disappearance at various incubation time points is presented in Figure 1. In general, starch disappearance was higher ($P < 0.001$) in defaunated (40.7%) than in faunated (35.0%) rumen fluid and was higher ($P = 0.001$) in the tube (39.4%) than in the bag (36.2%). The starch disappearance of the HVC was not different ($P > 0.05$) from that of the LVC (37.2 and 38.5%, respectively). A significant interaction between inoculum and incubation time was present ($P < 0.001$). Starch disappearance after 4, 6, and 12 h of incubation was significantly lower ($P = 0.043$, $P = 0.003$ and $P < 0.001$, respectively) in treatment FF than in DF. Starch disappearance after 2 and 24 h was not different ($P > 0.05$) between treatments FF and DF. However, this interaction between inoculum and incubation time differed for the tube and bag, as indicated by the significant ($P < 0.001$) 3-way interaction protozoa × incubation type × incubation time. Also, protozoa × corn source × time interaction was significant ($P = 0.003$). No other significant ($P > 0.05$) 3-way interactions were found. The starch disappearance data as affected by these significant 3-way interactions are presented in Table 1. In absence of protozoa, starch disappearance from the bag was higher after 6 and 12 h incubation compared with presence of protozoa ($P = 0.002$ and $P < 0.001$, respectively), but no differences ($P > 0.05$) between absence or presence of protozoa in the tube occurred. Starch disappearance in presence of protozoa after 12 h of incubation was lower ($P = 0.002$) in the bag (52.3%) than in the tube (66.4%). After shorter incubation periods (2, 4, or 6 h), starch disappearance from the bag was

Figure 1. Starch disappearance from the tube (A), and from the nylon bag (B). Symbols: defaunated dent ($\triangle$), defaunated flint ($\diamond$), faunated dent ($\blacktriangle$), and faunated flint ($\blacklozenge$).

only numerically smaller than that from the tube. In absence of protozoa, no differences ($P > 0.05$) between bag and tube were present. Finally, after 12 h of incubation, disappearance of starch from LVC was higher ($P = 0.007$) than that from HVC with faunated fluid (65.8 and 52.9%, respectively), but not with defaunated fluid ($P > 0.05$).

The estimated lag time and fractional degradation rate of starch (k_d), as well as the calculated extent of degradation using the Dhanoa et al. (1999) equation and assuming a fractional rumen passage rate (k_p) for these starch sources of 6.0%/h, are presented in Table 2. Of the main effects, only inoculum significantly affected lag time. Estimated lag time was higher ($P = 0.043$) in presence (4.6 h) than absence (3.6 h) of protozoa. Interactions were not significant ($P > 0.05$). Also, the main effects and 2-way interactions for k_d of starch and extent of degradation were not significant ($P > 0.05$).

DISCUSSION

In this study, 2 corn varieties were used, viz. a high vitreous endosperm corn (flint type) and a low vitreous endosperm corn (dent type). The flint corn tended to have a longer lag time than dent, which is in agreement with in situ work of Philippeau and Michalet-Doreau (1997) who found only a lag time with high vitreous flint corn. The 2 corn sources used in the present experiment did not differ significantly in starch disappearance, and there was no interaction with incubation type (bag or tube) and presence or absence of protozoa. Only at 12 h incubation of high vitreous corn, the faunated inoculum gave a lower starch disappearance than the defaunated inoculum, which is in contrast with our hypothesis (discussed later). An explanation for this lack of difference between the 2 corn varieties is the relatively high particle size. In this experiment, kernels were ground over a 3-mm sieve, whereas in general, particle sizes of 0.5 to 1.0 mm are used for in vitro incubations. This relatively large particle size can cause a high variation in vitreousness within the weighed

Table 1. Starch disappearance (expressed as % of starch at 0 h) after different incubation times as affected by inoculum (faunated, FF or defaunated, DF), incubation type (bag or tube), and corn source (low vitreous corn, LVC or high vitreous corn, HVC)

Incubation time, h	FF		DF		FF		DF	
	Bag	Tube	Bag	Tube	LVC	HVC	LVC	HVC
2	3.9	4.4	-1.2	5.2	5.4	2.9	3.6	0.4
4	3.6 ^a	11.5 ^{ab}	13.2 ^{ab}	15.8 ^b	7.6	7.4	17.2	11.2
6	10.9 ^b	14.8 ^{ab}	25.2 ^a	18.2 ^{ab}	12.7	12.9	21.0	22.4
12	52.3 ^b	66.4 ^a	71.9 ^a	70.1 ^a	65.8 ^a	52.9 ^b	68.5 ^a	73.5 ^a
24	91.7	90.2	90.9	97.9	98.8	92.1	93.4	95.3

^{a,b}Means in the same row within 3-way interaction with different superscripts differ ($P < 0.05$). The SEM is 2.05.

Table 2. Lag time (lag) and fractional degradation rate (k_d) with asymptotic standard deviation (SD), and calculated extent of degradation (E) as affected by inoculum (faunated, FF or defaunated, DF), incubation type (bag or tube), and corn source (low vitreous corn, LVC or high vitreous corn, HVC)

Item	FF				DF			
	Bag		Tube		Bag		Tube	
	LVC	HVC	LVC	HVC	LVC	HVC	LVC	HVC
lag, h	5.2	5.0	3.4	4.9	3.1	3.3	2.8	5.0
SD	0.32	0.76	0.77	0.67	0.52	0.37	0.98	0.55
k_d , %/h	13.4	10.1	12.0	13.3	11.3	14.7	12.0	17.9
SD	1.34	1.75	2.90	2.56	1.60	1.89	3.21	3.91
E ¹	0.506	0.465	0.544	0.514	0.542	0.583	0.564	0.555

¹Extent of ruminal degradation of starch calculated as $k_d \exp(-k_p \text{ lag}) / (k_d + k_p)$ (Dhanao et al., 1999), where k_p is fractional rate of passage and set at 6.0%/h.

sample. Another explanation is the relatively small difference in vitreousness between the used corn varieties (68 and 81%). Philippeau et al. (1999) showed a variation of 39 to 79% vitreousness between different corn grain varieties.

The lower starch disappearance from the nylon bags compared with the tubes in our study helps to explain the lower starch degradation based on in situ data compared with in vivo data for slowly degradable starch sources as observed by Offner and Sauvant (2004). The underestimation of starch degradation in the nylon bag with the faunated inoculum seems to be caused by the slower degradation during h 4 to 12 (Figure 1). This difference is however only significant at 12 h due to variation between the duplicated incubations. Possibly, in vivo ruminal degradation of large cornstarch particles occurs at a faster rate than estimated by the nylon bag method. This would mean that cornstarch is more rapidly available for ruminal microbes, stimulating microbial growth and VFA production more than assumed previously. This also indicates that cornstarch has higher potential in lowering ruminal pH than generally is assumed.

Although starch disappearance in nylon bags started slower than in the tube, the results do not confirm the hypothesis that the deviation between in vivo and in situ degradation as noted by Offner and Sauvant (2004) is due to limited protozoal access to the nylon bag. At 6 and 12 h of incubation, defaunation even increased nylon bag disappearance (Table 1). Also, the disappearance of the high-vitreous corn inoculated with defaunated rumen fluid was numerically higher, and at 12 h significantly higher, than that with faunated rumen fluid. This was in contrast with our hypothesis that the presence of protozoa will improve the degradation of the protein matrix surrounding the starch, allowing faster access and degradation of the starch.

Although all entodiniomorphid protozoa engulf starch grains to ferment it more slowly, the uptake rate

varies between species. The larger entodiniomorphid protozoa engulf starch grains at a low but constant rate for several hours, whereas in the smaller *Entodinium* spp., initially starch grains are taken up rapidly and then much more slowly as the cell contains more starch (reviewed by Williams and Coleman, 1997). The degradation rate of starch inside the protozoa may be limited because amylolytic enzyme activities are subject to negative feedback by the end products of the degradation of starch. Thus it may be expected that inside the bag, where only smaller protozoa can enter, initial starch uptake is faster than in the tube, where large protozoa contribute to starch uptake and degradation. Uptake of starch by protozoa prevents its immediate and rapid fermentation by bacteria. Specific amylase activity of mixed rumen protozoa has been observed to be higher (Mendoza et al., 1995) or lower (Martin et al., 1999) than that in mixed bacteria. In an in vitro experiment, Nagaraja et al. (1986) observed that fermentation of starch with rumen fluid devoid of protozoa (by means of low speed centrifugation) resulted in decreased pH and increased VFA concentrations compared with strained rumen fluid containing protozoa, indicating a moderating effect of protozoa on starch fermentation. Mendoza et al. (1993) showed that the presence of protozoa decreased in situ corn or sorghum starch degradation. These findings are in line with our results that the presence of protozoa reduces starch disappearance in the bag at 6 and 12 h incubation. Because this effect is not present in the tube, the different pattern of starch engulfment between small and large protozoa, and the compensatory effect of amylolytic bacteria, may have a more pronounced role. In contrast, Fondevilla and Dehority (2001) reported an increase in cornstarch disappearance in vitro with a coculture of *E. exiguum* and bacteria compared with bacteria only. An important difference between our study and that of Fondevilla and Dehority (2001) is the fact that the latter used only one protozoa species. The rumen has a diverse protozoal

population, which may have a greater influence on bacterial growth than the single cultured *E. exiguum*. Overall, our findings indicate that the protozoal degradation of the protein matrix surrounding starch is of relatively minor importance compared with the role of protozoa in slowing starch degradation.

Our in vitro results do not agree with the in vivo findings of Veira and Ivan (1983) in sheep, but are in line with in vivo results in cattle reported by Nagaraja et al. (1992) and in sheep by Mendoza et al. (1993). Veira and Ivan (1983) observed improved ruminal starch degradation in faunated sheep compared with defaunated sheep. In this experiment, diets low in crude protein (11.3%) were fed. The defaunated sheep had very low rumen ammonia levels, and these low ammonia levels may have impaired microbial activity. Indeed Mendoza et al. (1995) observed an interaction between the level of urea added and protozoal presence on in vitro starch degradation. The presence of protozoa increased starch degradation when urea levels were low, but starch degradation was not affected by presence or absence of protozoa at high urea levels. In the present trial, tryptone was added to the medium as a supplemental N source for bacteria to prevent N shortage. Nagaraja et al. (1992) found an increase in bacterial numbers and VFA concentration in defaunated steers on a high grain diet and concluded that ciliate protozoa moderate the rate of substrate fermentation in the rumen. Mendoza et al. (1993) observed a lower (35%) in situ rate of starch degradation and a decreased (9%) extent of ruminal starch degradation in faunated sheep compared with defaunated sheep. These latter results are in line with the concept that protozoa slow starch fermentation in the rumen. Apparent differences in starch degradation in studies with sheep may also occur because of particular dynamics of changes in rumen microbial populations as compared with cattle. Using denaturing gradient gel electrophoresis to elucidate population changes indicated that sheep have a dynamic rumen microbial community, whereas bacterial populations in cattle are much more stable both within and between animals across a range of diets (A. Klieve, Queensland Department of Primary Industries and Fisheries, Yeerongpilly, Australia, personal communication).

In conclusion, in presence of protozoa, starch disappearance rate is slower inside nylon bags than outside nylon bags. Absence or presence of protozoa did not affect starch degradation outside the bag. These results help to explain the underestimation of starch degradation based on the in sacco procedure when compared with in vivo data on slowly degradable starch sources.

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