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Presence of carbohydrate-digesting enzymes throughout the digestive tract of sheep

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Abstract: The activity of enzymes that digest carbohydrates at various points in the gastrointestinal tract of sheep was examined. In addition, weight and contents of digestive tract segments were examined. The experiment was performed on six adult ewes fed an 85% hay diet. The morphometric measurements revealed that the alimentary tract constituted 32% of the sheep's body mass and wet rumen contents constituted 70% of wet mass of the total digestive tract content. The protozoal population in the rumen varied from 37.2×10^4 to 54.0×10^4/mL rumen fluid. In the examined samples, ciliates were identified as belonging to the family Ophryoscolecidae, genera *Entodinium*, *Diplodinium*, and *Ophryoscolex*, and family Isotrichidae, genera *Isotricha* and *Dasytricha*. Degradation of various carbohydrates by digesta revealed that the fastest digestion rate for plant cell wall carbohydrates and inulin occurred in the reticulum, whereas for starch and chitin, rates of digestion to reducing sugars were the highest in the small intestine. The pH and redox potential at various points in the digestive tract varied from 2.8 to 7.7 and from –72.1 to –289.2 mV, respectively.

Key words: Carbohydrate digestion, protozoa, digestive tract, sheep

1. Introduction

Sheep breeding is one of the oldest professions closely associated with human civilization. The group of livestock called ruminants has a great utility. Sheep breeding yields meat and lambs, and, to a lesser degree, milk, wool, and hide. The physiology and morphology of sheep reflect adaptations to particular ecological niches. Based on food preference, sheep are herbivorous animals that prefer food rich in cellulose, i.e. 'grass/roughage eaters' (1). The most important feature of ruminants is the four-chambered stomach in the front of the gastrointestinal tract. Digestion of plant material occurs in the largest chamber, in the rumen, thanks to symbiotic microorganisms belonging to three taxonomic groups: bacteria, fungi, and protozoa (2). Among the protozoa, the most abundant and most important are the ciliates belonging to the family Ophryoscolecidae, followed by the family Isotrichidae. Protozoa differ in food preferences. Representatives of Ophryoscolecidae prefer insoluble carbohydrates, e.g., starch and cellulose, whereas Isotrichidae ciliates prefer soluble polysaccharides and do not utilize cellulose (3).

Numerous studies have assessed digestive processes in the rumen, but relatively few provided information about digestion later in the digestive tract of ruminants. The goal of this work was to characterize degradation of carbohydrates in the full gastrointestinal tract of adult sheep.

2. Materials and methods

2.1. Animals, feeds, and feeding

Six adult Polish Merino sheep with an average body mass of 55.3 kg were kept in separate pens. Their diet consisted of high-quality ingredients: 1560 g of meadow hay (which was collected on approximately 20 May, before heading), 250 g of ground barley, and 20 g of vitamin-mineral premix (Polfamix OK, Trouw Nutrition Polska, Grodzisk Mazowiecki, Poland), as shown in Table 1. The daily ration was divided into two equal parts and fed at 0700 and 1900 hours. Water was available ad libitum. Feed analyses were conducted in the Laboratory of Chemistry, The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, using AOAC methods (4). Cellulose was calculated as ADF – ADL according to Rinne et al. (5).

2.2. Measurements of the length and weight of the digestive organs

Animals were weighed and then slaughtered 3 h after the morning feeding according to standard procedures. Immediately after slaughtering, the entire digestive tract of sheep was removed and the connective tissues and lipids were removed carefully. Particular organs in the digestive tract (the stomach including the rumen, reticulum, omasum, and abomasum, as well as the small intestine, cecum, and large intestine) were isolated by ligating these

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Table 1. Composition of the ration.

Item	% of dry matter
Meadow hay	85.22
Ground barley	13.71
Vitamin-mineral premix[1]	1.11
Nutrient analysis	
Organic matter	92.05
Crude protein	18.41
N-free extract	30.03
Crude fiber	28.02
NDF	43.01
ADF	22.04
ADL	4.41
Cellulose	17.62

[1]Premix contents per kg: Ca - 230 g, P - 125 g, Na - 60 g, Mg - 65 g, Fe - 0.5 g, Co - 0.015 g, Mn - 3 g, Zn - 2.5 g, I - 0.01 g, Se - 0.003 g, vitamin A - 300,000 IU, vitamin D3 - 30,000 IU, and vitamin E - 1.5 g; NDF - neutral detergent fiber, ADF - acid detergent fiber, ADL - acid detergent lignin.

organs with a string to prevent movement of the contents. The reticulum was separated from the stomach by binding it at the place of stenosis of the reticulo-ruminal fold and reticulo-omasal orifice. All procedures of the separation process were done extremely carefully to prevent movement of digesta contents. The presence of feces in the rectum showed that slaughter stress did not cause content evacuation from the large intestine, so it can be assumed that the obtained measurements can be applied to live animals. The digestive organs were weighed, emptied, and weighed again to aim to calculate the mass of each of the digestive components by difference. Three samples of digestive tract contents (DTCs) were collected from each organ to determine dry matter and hydrolytic activities. The pH and the redox potential (Eh) were measured immediately with a pH meter (model 7011, ChemLand, Poland) equipped with respective electrodes. Contents of each organ were frozen and stored at –80 °C for future analysis.

2.3. Extraction and measurement of enzymatic activity

The degradation rate of plant carbohydrates (cellulose, starch, inulin, pectin, xylan) as well as microbial carbohydrates (chitin) was determined by a common method relying on the quantification of reducing sugars released from the appropriate substrate following incubation of substrates with the enzymatic fraction of DTCs. The digestive tract enzymes were extracted by the procedure of Huhtanen and Khalili (6). Briefly, digesta (2 g, wet weight) were incubated for 3 h with 2.5 mL of CCl_4 and 20 mL of 1% phosphate buffer (pH 6.0) in the presence of 1 mL of lysozyme solution (50 mg/mL). The extract obtained was centrifuged at 11,000 × *g* for 30 min at 4 °C, and the supernatant was collected and used as an enzymatic fraction of DTC for enzymatic studies. The reaction mixture consisted of 0.4 mL of substrate, 0.4 mL of enzymatic fraction of DTC, and 0.2 mL of phosphate buffer (pH 6.0) after the mixture had been incubated for 1 h at 40 °C, and the reaction was stopped by addition of 1.2 mL of salicylic acid reagent as described by Miller et al. (7). The enzymatic fraction of DTC without substrate and the substrate without the enzymatic fraction of DTC were concomitantly incubated as controls. Absorbance was measured at 560 nm using a Hach Lange DR 6000 UV VIS spectrophotometer (Hach Company, Loveland, CO, US). A 0.2% solution of pectin (Sigma-Aldrich P9135) as well as 2% solutions of CMC (Sigma-Aldrich C5678), starch (Sigma-Aldrich S5651), inulin (Orafti HPX), xylan (Sigma-Aldrich X4252), and colloidal chitin were used as substrates. The colloidal chitin was prepared from commercial chitin (Sigma-Aldrich C7170) according to Shen et al. (8). The concentration of the reducing sugars released during the incubation of the enzymatic fraction of DTC with the examined substrate was calculated by comparing it with the absorbance of galacturonic acid, glucose, fructose, xylose, and N-acetylglucosamine standards, respectively, and was expressed as equivalent of mentioned sugar per gram of dry matter of digesta content per minute. The DM of digesta contents was determined by drying samples at 105 °C for 48 h to constant weight. The total activity of particular segments of the digesta contents was calculated with respect to the total activity of DTCs.

2.4. Protozoa

Samples of rumen fluid were withdrawn immediately after the slaughter of the sheep. To obtain a representative sample, rumen fluid was taken from the dorsal and ventral sacks of the rumen and thoroughly mixed. Large particles of food were removed by filtration through two layers of medicinal gauze (pore size: 1 mm). The samples of rumen fluid for counting protozoa (5 mL) were fixed with an equal volume of 4% aqueous formaldehyde solution. The protozoa were identified according to Dogiel (9) and counted in the manner described by Miltko et al. (10).

2.5. Calculations

The measurements were performed in triplicate for each sample taken from all parts of the digestive tract. The obtained results were presented as average value ± standard deviation (SD).

3. Results

Body mass of the 18-month-old ewes averaged 55.3 kg and varied from 54.6 kg to 56.8 kg. These values matched the data presented by Martyniak and Rzepecki (11). Mass and length of segments and contents (wet) of the gastrointestinal tract are presented in Table 2. The mass of the digestive tract plus contents constituted 32% of the total body mass of the animal, while the stomach (rumen, reticulum, omasum, and abomasum) constituted 25% of total body mass of the sheep and 78% of the total mass of the gastrointestinal tract. The largest part of the stomach was the rumen; it represented 75% of its mass. The average wet mass of contents of the total digestive tract was 12.4 ± 1.68 kg. The mass of the rumen content accounted for 70% of the total mass of gastrointestinal tract, while the remaining parts of the stomach constituted only 13% of the total mass. The longest segment in the digestive tract was the small intestine, which accounted for 80% of the total length of the intestines, whereas the cecum accounted for less than 1%. Characteristics of the contents of the digestive tract are shown in Table 3. The values of pH varied among the different parts of the digestive tract of sheep. The pH of abomasal contents was the lowest (2.5–3.1), whereas in the small intestine it was the highest (7.5–7.9). Digesta in the forestomach (rumen, reticulum, omasum) had pH values ranging from 5.8 to 6.7, while for the cecum and large intestine they were near neutrality, varying from 6.9 to 7.3. Redox potential of digesta varied ranged from –72.1 to –289.2 mV. The Eh of the forestomachs, cecum, and large intestine was lower than in the abomasum and small intestine. The percentage of the DM of the content differed among organs (Table 3). The highest value was in the omasum (16.2%), with the lowest in the small intestine (6.5%). The total of DM of digesta among organs ranged from 35.6 to 1114.3 g. Mass of DM per organ was the lowest in the cecum at 2.3%, and the highest was in the

Table 2. The mass and length of the particular segments of the digestive tract of sheep.

Organ	Mass (kg)		Length (m)
	Content + tissue	Content	
Rumen	10.4 ± 0.52	8.7 ± 0.78	-
Reticulum	0.8 ± 0.15	0.4 ± 0.06	-
Omasum	1.3 ± 0.09	0.4 ± 0.07	-
Abomasum	1.4 ± 0.15	0.8 ± 0.13	-
Small intestine	1.2 ± 0.32	0.6 ± 0.31	24.4 ± 2.73
Cecum	0.6 ± 0.33	0.4 ± 0.12	0.3 ± 0.13
Large intestine	2.0 ± 0.31	1.1 ± 0.21	5.9 ± 1.04

Means ± SD.

Table 3. Characterization of the contents of the digestive tract of sheep.

Organ	pH	Eh (mV)	% DM	DM per organ (g)
Rumen	6.0 ± 0.2	–261.1 ± 8.3	12.8 ± 1.5	1114.3 ± 222.3
Reticulum	6.6 ± 0.1	–260.4 ± 8.0	9.5 ± 1.7	39.3 ± 11.8
Omasum	6.2 ± 0.2	–172.8 ± 14.0	16.2 ± 1.6	63.3 ± 19.2
Abomasum	2.8 ± 0.3	–72.1 ± 10.3	10.3 ± 1.3	83.0 ± 27.7
Small intestine	7.7 ± 0.2	–127.2 ± 10.4	6.5 ± 1.4	52.6 ± 18.5
Cecum	7.1 ± 0.2	–276.1 ± 12.0	10.3 ± 2.5	35.6 ± 9.4
Large intestine	7.1 ± 0.2	–289.2 ± 16.4	10.4 ± 2.2	123.4 ± 36.1

Means ± SD.

rumen at 73.7% of the total mass of dry matter of the full digestive tract.

Composition of rumen protozoa was similar among all ewes. The population density and species distribution of the members of families Ophryoscolecidae and Isotrichidae are shown in Table 4. The most numerous group of protozoa belonging to Ophryoscolecidae was *Entodinia*, which constituted 88.0% of the total number of ciliates in the rumen. The concentration of the particular species from this genus was not determined. The genus *Diplodinium* was represented by four species: *Anoplodinium denticulatum* (4.4%), *Eudiplodinium maggii* (1.8%), *Diploplastron affine* (0.9%), and *Polyplastron multivesiculatum*, at 0.9% of the total number of rumen protozoa. The mean concentration of *Ophryoscolex caudatus* was (0.6 ± 0.2) × 10^4/mL rumen fluid. The members of family Isotrichidae constituted only 2.7% of the total protozoal population.

Hydrolytic activities of contents of various segments of the digestive tract are presented in Table 5. The obtained results revealed degradation of all tested carbohydrates in the whole digestive tract, but at different rates. The mean value of total hydrolytic activity was 5.9 ± 1.3 μmol released equivalents of reducing sugars/g DM of DTC per minute. The highest degradation rates for starch and chitin were detected in the small intestine, whereas for inulin, pectin, cellulose, and xylan such rates were detected in the reticulum. Among the examined carbohydrates, the fastest digestion was for starch, where the degradation rate varied from 3.7 ± 0.6 μmol glucose/g DM of DTC per minute (in the omasum) to 36.9 ± 8.4 μmol glucose/g DM of DTC per minute (in the small intestine). The total hydrolytic activity in the rumen constituted 75% of the total activities of the DTC, whereas in the others segments degradation rate varied between 2% and 6% of total DTC activity.

Table 4. The concentration of protozoa (×10^4 per mL of rumen fluid) and species distribution in the rumen of sheep.

Family	Species	Population density
Ophryoscolecidae	*Entodinium* spp.	40.2 ± 7.0 (88.0%)
	Anoplodinium denticulatum	2.0 ± 0.5 (4.4%)
	Eudiplodinium maggii	0.8 ± 0.1 (1.8%)
	Diploplastron affine	0.4 ± 0.2 (0.9%)
	Polyplastron multivesiculatum	0.4 ± 0.1 (0.9%)
	Ophryoscolex caudatus	0.6 ± 0.2 (1.4%)
Isotrichidae	*Dasytricha prostoma*	0.6 ± 0.1 (1.4%)
	Isotricha spp.	0.6 ± 0.1 (1.3%)
Total protozoa		45.6 ± 8.4

Means ± SD.

Table 5. The degradation rate of particular carbohydrates by enzymatic fraction of the digestive tract contents (μmol of released monosaccharide/g DM of content per minute).

Organ	Carbohydrates					
	Starch	Inulin	Pectin	Cellulose	Xylan	Chitin
Rumen	6.4 ± 2.0 (64%)	4.8 ± 0.8 (75%)	4.3 ± 0.6 (73%)	7.7 ± 2.3 (81%)	11.7 ± 2.3 (81%)	1.2 ± 0.2 (76%)
Reticulum	8.6 ± 1.5 (3%)	5.8 ± 2.0 (3%)	6.3 ± 0.8 (4%)	11.6 ± 0.8 (4%)	15.3 ± 2.0 (4%)	1.4 ± 0.1 (3%)
Omasum	3.7 ± 0.6 (2%)	3.9 ± 1.5 (4%)	2.8 ± 0.2 (3%)	4.8 ± 1.4 (3%)	11.1 ± 2.6 (4%)	0.9 ± 0.2 (3%)
Abomasum	4.8 ± 1.6 (4%)	4.0 ± 0.9 (5%)	4.1 ± 1.2 (5%)	4.3 ± 1.6 (3%)	4.7 ± 1.8 (2%)	0.8 ± 0.3 (4%)
Small intestine	36.9 ± 8.4 (18%)	5.5 ± 2.0 (4%)	5.7 ± 0.8 (5%)	5.0 ± 1.5 (3%)	6.5 ± 1.7 (2%)	2.1 ± 0.6 (6%)
Cecum	6.6 ± 1.9 (2%)	4.3 ± 1.5 (2%)	4.1 ± 0.6 (2%)	4.1 ± 1.0 (2%)	6.8 ± 2.0 (2%)	1.0 ± 0.1 (2%)
Large intestine	6.3 ± 1.0 (7%)	3.7 ± 0.8 (7%)	4.4 ± 0.5 (8%)	3.6 ± 0.4 (4%)	5.8 ± 1.7 (5%)	0.9 ± 0.2 (6%)

Means ± SD. Values in parentheses are percentages of hydrolytic activity from activity of total activity of the digestive tract.

4. Discussion

The structure of the gastrointestinal tract is largely determined by the type of food. Ruminant animals have developed complex adaptations to a low-quality diet. One of them is the presence of the forestomach, which gives them the opportunity of pregastric digestion and fermentation of carbohydrates. Degradation of food components in pregastric chambers takes place due to enzymes synthesized by symbiotic microorganisms. In the remaining segments of the gastrointestinal tract, digestion is assisted by the animal's own enzymes and by enzymes of microorganisms. The goal of this study was to compare the digestion rate of storage carbohydrates (starch and inulin) and structural carbohydrates (pectin, xylan, cellulose, and chitin) in various segments of the digestive tract of sheep. Total mass of the digestive tract (content + tissues) and the mass of the contents alone constituted 32% and 22% of the total body mass of the animals, respectively. The ratio of the total stomach mass (rumen, reticulum, omasum, and abomasum) to the total mass of the intestines was about 3.7. The obtained results were much higher than those described by Yıldırım et al. (12). In adult ruminants the rumen is functionally integrated with a reticulum; therefore, it is commonly called the reticulo-rumen (1). In the current study the mass of the reticulo-rumen contents exceeded 73% of the total mass of the DTCs, in accordance with the results described by Sekine et al. (13). Morphometric studies revealed that the small intestine accounted for 80% of the total length of the intestines, whereas the large intestine accounted for about 19%. These proportions in length were typical for grass-eating ruminants (1). However, it should be noted that animals included in this group may have different anatomies in the individual segments of their digestive tract. For example, the length of the large intestine in cows and sheep is similar (14); however, the construction of the colon shows some differences. In cattle it is distinguished by 1.5 to 2 turns centrifugally, whereas in sheep it is 3. These differences affect the resorption of water, and this may have an influence on dry matter and pH of digesta contents.

The digestive tract of ruminants provides the best conditions for microbial populations by maintaining stable temperature, pH, and potential redox. Mean pH values in the contents of the digestive tract were similar to results obtained by Wheeler and Noller (15). Rumen pH varied from 5.8 to 6.2, typical for this part of digestive tract (16,17) with forage-based diets. In the reticulum the pH was significantly higher in comparison to the rumen, in accordance with the results obtained by Kimura et al. (18). This difference is presumably due to the presence of more saliva secreted by the animal to the reticulum (19). The pH of the omasum was similar to the pH of the rumen due to absorption and exchange of cations and anions in this part of the forestomach (20). The pH in the abomasum was low due to secretion of hydrochloric acid by gastric glands, and was typical for ruminants. The increase of pH up to 7.7 in the small intestine is caused by the secretion of the strongly alkaline pancreatic juice. For the other parts of the digestive tract (cecum and large intestine) the pH values were nearly neutral, similar to results obtained by Rezaeian et al. (16) for forage-fed ruminants.

The mean values of potential redox (Eh) of the particular segments of the digestive tract obtained in the current study were similar to the results described by Marounek et al. (21). Differences in Eh are related to activity of anaerobic and aerobic microbiota, being greatest for anaerobes when the potential redox ranges from 50 to –400 mV, while for aerobes the optimum range is 400 to –200 mV (22).

Dry matter of the DTC matched the data described by Rezaeian et al. (16). The highest value of dry matter was detected in the omasum due to the absorption of water occurring in this forestomach (20). The same mechanism was observed in the large intestine, where water resorption occurred in the spiral colon (14).

The rumen protozoa are sensitive to changes in their environment and have specific nutritional requirements. On roughage diets, their biomass is lower compared to concentrate diets (23). However, a high level of storage carbohydrates (e.g., starch) in ration feeding could induce mild acidosis and lead to defaunation (24). The population density of ciliates and their composition in the rumen were similar to results described by the other authors (17). The identified protozoa possess various hydrolytic enzymes and can digest diverse types of carbohydrates. The main group of protozoa detected in this part of stomach was *Entodinia*, which constituted 88.0% of the total number of ciliates in the rumen. These protozoa prefer concentrate diets, food rich in starch (3). The next group of protozoa detected were ciliates that also synthetized enzymes for degrading structural carbohydrates. The fibrolytic species identified in the rumen were *Anoplodinium denticulatum*, *Eudiplodinium maggii*, *Diploplastron affine*, *Polyplastron multivesiculatum*, and *Ophryoscolex caudatus*. The less numerous members in the rumen were holotrich ciliates, *Dasytricha ruminatium* and *Isotricha* spp., that digest soluble carbohydrates. Ruminants do not synthesize enzymes that degrade plant carbohydrates such as cellulose, xylan, pectin, and inulin, which are the main components of their diet, as well as chitin, a microbial carbohydrate. Nevertheless, the energy stored in these carbohydrates is utilized by ruminants. This occurs due to the action of the digestive enzymes of microbial origin. Bacteria, fungi, and protozoa that live in the gastrointestinal tract possess a number of adaptations to the digestion of plant

material, which are specific to each taxonomic group. The main place of activity of these microorganisms is the rumen; however, anaerobic fungi (16) and bacteria (25) can colonize and degrade fiber components in all parts of the digestive tract. The use of lysozyme in enzymatic studies provides a breakdown of the microorganisms' cell wall. This procedure is widely used by researchers in these types of experiments and is an alternative to efficient but time-consuming sonication (26). The enzymatic studies revealed that all carbohydrates examined were digested throughout the entire length of the digestive tract. Moreover, it was proved that xylan, cellulose, pectin, and inulin were effectively digested in the reticulum. Two reasons for this may be taken into account. First, the proportion of small particles was very high in the reticulum contents, and consequently a large surface area was available for microbial attack (27). The second explanation could reflect greater diversity in composition of the microbial population in the reticulum contents than in the rumen contents (28). Starch, in contrast to other plant carbohydrates, is hydrolyzed by enzymes originating from microbes and animals. Amylolytic activity was the highest in the small intestine due to the secretion of α-amylase by the pancreas. Chitin was most actively digested in the small intestine, perhaps due to a relatively large concentration of this fungal carbohydrate at this point of the digestive tract (16). However, total hydrolytic activity of the DTC proved that the rumen is the most important site for digestion of fiber-based diets in the ruminants. This matches suggestions by Simunek et al. (29).

Digestive organ size, dry matter and pH of digesta contents, and characterization of the digestive processes may vary with breed of sheep, age, sex, condition of the animals, and especially different types of feeds (12,30). Our results show that Polish Merino ewes can digest structural (pectin, cellulose, xylan, and chitin) as well as storage (starch, inulin) carbohydrates at all points of the gastrointestinal tract.

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