Biochemical properties and hydrolytic activity in the nutria (*Myocastor coypus*) digestive tract

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SUMMARY

Current understanding of site-specific digestion of carbohydrates in mammals is that whereas starch can be degraded by mammalian enzymes, other complex carbohydrates (e.g. pectin, inulin, xylan or cellulose) are typically digested by the enzymes of symbiotic microbes. To test whether the previously reported presence of complex carbohydrate-digesting enzymes in the small intestine of sheep represents actual small intestinal enzyme activity or just outflux of microbial enzymes from the forestomach, we applied the same methodology of isolating enzymes from gastrointestinal contents and applying them in vitro to test substrates in the nutria (*Myocastor coypus*), a hindgut-fermenting rodent without a forestomach. No enzymatic activity against carbohydrates was detected in the stomach, excluding an effect of coprophagy on the presence of the investigated enzymes. While – as expected – starch digestion was highest in the small intestine, and that of the other carbohydrates was highest in the caecum, there was nevertheless detectable enzymatic activity against pectin, inulin, xylan and cellulose in the small intestine. While further research is warranted, we suggest that these results indicate a certain degree of unspecific carbohydrase activity by small intestinal enzymes that plays no relevant role in vivo due to the short residence time of digesta in the small intestine.

KEY WORDS: *Myocastor coypus*, Enzymatic digestion, Carbohydrate, Microbial digestion, Starch, Cellulose
INTRODUCTION

Nutrias (*Myocastor coypus*) are hystricomorph rodents and strict herbivores with a possible preference for monocots and roots associated with water bodies (Willner et al., 1979; Abbas, 1991; Borgnia et al., 2000). Nutrias have a simple stomach and a large caecum (Wagner, 1963; Snipes et al., 1988). Plant fibre is fermented in the caecum (Marounek et al., 2005), and nutrias seemingly have a higher fibre digestion capacity than rabbits and several other rodents (Sakaguchi and Nabata, 1992; Hagen et al., 2019). Like other hystricomorph rodents, nutrias have a colonic separation mechanism of the ‘mucus trap’ type with a colonic furrow or groove that serves to retain microbial matter (Snipes et al., 1988; Takahashi and Sakaguchi, 2000). This material is periodically ingested as ‘soft faeces’ in the process of coprophagy or ‘caecotrophy’ (Takahashi and Sakaguchi, 1998).

Monogastric, hindgut-fermenting herbivorous mammals typically prevent microbial growth in the stomach by means of a low pH; afterwards, the ingested food is digested by enzymes produced by the animal itself in the small intestine (Karasov and Hume, 1997). Subsequently, digestion by microbial enzymes (‘fermentation’) occurs in the caecum and/or colon (Karasov and Hume, 1997; Stevens and Hume, 1998). This sequence is associated with distinct changes in the biochemical properties of the digesta (such as pH and redox potential) (Miltko et al., 2019), as well as in enzymatic activity. Here, we used the opportunity of a larger experiment on the effect of diet on nutria growth and fatty acid composition (Głogowski et al., 2010; Głogowski et al., 2018) to assess these measurements in the nutria. In particular, we were interested to know whether the comparatively high levels of activity of enzymes that break down various fibre fractions detected in the small intestine of sheep (Miltko et al., 2016) would also be present in the nutria (and hence represent a component of small intestine function) or not (and hence could be considered outflow from the forestomach in sheep).

MATERIALS AND METHODS

All animals were kept at a commercial nutria farm in Poland and slaughtered during routine procedures. The study was conducted with the approval of the Third Local Ethics Committee for Animal Experiments at the University of Life Sciences, Ciszewskiego 8, 02786 Warsaw.

We used five female nutrias aged 10-12 months with an average body weight of 5.2 ±0.6 kg. The diet, described in detail in Głogowski et al. (2010), consisted of steamed potatoes and fresh grass or clover, estimated at 125 g crude protein and 152 g crude fibre per kg dry matter. Animals were housed indoors, in open pens without water pools. Slaughter took place in accordance with EU regulations by electrical stunning and bleeding (Głogowski et al., 2018), after which the entire gastrointestinal tract (GIT) was immediately dissected and string ligations were placed between the stomach, small intestine, caecum and colon, to prevent movement of contents between these sites. Subsequently, these organs were separated from the mesenteries and adhering adipose tissues, and the organs were measured for length and weighed full and empty. Results for GIT dimensions as well as tissue and content weight are given in Głogowski et al. (2018).

Contents of each of the GIT sections were collected. A portion of the content samples was frozen and stored at –80°C for later analysis. Another portion was filtered through four layers of gauze (1mm pore
size), and the pH and redox potential (Eh) were measured in the resulting fluid fraction (pH meter 7011, ChemLand, Stargard Szczeciński, Poland).

The dry matter (DM) of the GIT contents was determined by drying the samples at 105°C for 48 h to constant weight. The degradation rate of plant carbohydrates (starch, cellulose, inulin, pectin, and xylan) was determined by quantifying reducing sugars released from the respective substrate following incubation with the enzymatic fraction of the GIT contents. The GIT content enzymes were extracted by the procedure of Huhtanen and Khalili (1992), as also used by Miltko et al. (2016). Two grams of digesta dry weight were incubated with 2.5 mL of CCl4 and 20 mL of 1% phosphate buffer (pH 6.0) in the presence of 1 mL of lysozyme solution (50 mg/mL). After three hours, the material was centrifuged at 11,000 × g for 20 min at 4°C, and the supernatant was collected and used as the enzymatic fraction characteristic of the respective GIT contents.

The reaction mixture for releasing the reducing sugars consisted of 0.5 mL of test substrate, 0.3 mL of enzymatic fraction of GIT contents, and 0.2 mL of phosphate buffer (pH 6.0). After the mixture had been incubated for 1 h at 40°C, the reaction was stopped by addition of 1.25 mL of salicylic acid reagent (Miller et al., 1960). The enzymatic fraction of GIT contents without substrate and the substrate without the enzymatic fraction of GIT contents were concomitantly incubated as controls.

Test substrates were solutions of 0.2% starch (Sigma-Aldrich S5651), 0.1% pectin (Sigma-Aldrich P9135), 0.1% inulin (Orafti HPX), 0.4% xylan (Sigma-Aldrich X4252) and 1% carboxymethylcellulose (Sigma-Aldrich C5678). All substrates used in this study had been used in previous experiments, in which they showed high degree of microbiological digestibility (Miltko et al., 2016).

Absorbance was measured at 560 nm using a Hach Lange DR 6000 UV VIS spectrophotometer (Hach Company, Loveland, CO, US). The concentration of the reducing sugars released during the incubation of the enzymatic fraction of GIT contents with the test substrates was calculated by comparing it with the absorbance of galacturonic acid, glucose, fructose, and xylan standards, respectively. The concentration of these sugars measured in the pure enzymatic fraction of GIT contents without substrate and the pure test substrate was subtracted from that measured in the incubation test. The result was expressed as equivalent of the respective sugar per gram of dry matter of GIT contents per minute.

All measurements were performed in triplicate. Results are presented as means ± standard deviation. Statistical comparisons between GIT sections were performed in R (R Core Team, 2020). After confirming normal distribution of the data using the Shapiro-Wilk test, we used the ez package (Lawrence, 2011) to perform a repeated-measurements ANOVA followed by Bonferroni post hoc tests to adjust for multiple comparisons. The significance level was set to 0.05.

RESULTS

The pH was lowest, and the redox potential and dry matter concentration highest in the stomach contents; the dry matter concentration was lowest in the small intestine contents (Table 1). Whereas the redox potential for the stomach indicated an aerobic environment, that of the other GIT sections indicated an anaerobic environment, with the lowest Eh values in the caecum (Table 1).
Table 1.
Characterization of the gastrointestinal digesta of nutrias with respect to pH, redox potential (Eh) and concentration of dry matter (DM)

<table>
<thead>
<tr>
<th>GIT section</th>
<th>pH</th>
<th>Eh (mV)</th>
<th>DM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>3.0 ± 0.7&lt;sup&gt;A&lt;/sup&gt;</td>
<td>103.4 ± 17.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>22.2 ± 1.9&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>7.7 ± 0.2&lt;sup&gt;B&lt;/sup&gt;</td>
<td>-191.2 ± 22.6&lt;sup&gt;B&lt;/sup&gt;</td>
<td>10.5 ± 1.8&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>Caecum</td>
<td>5.7 ± 0.3&lt;sup&gt;C&lt;/sup&gt;</td>
<td>-264.2 ± 33.1&lt;sup&gt;C&lt;/sup&gt;</td>
<td>14.9 ± 1.0&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>Colon</td>
<td>6.0 ± 0.3&lt;sup&gt;C&lt;/sup&gt;</td>
<td>-228.4 ± 46.9&lt;sup&gt;BC&lt;/sup&gt;</td>
<td>17.6 ± 1.8&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Within a column, values with different superscripts (A,B,C) differ significantly (RM-ANOVA, Bonferroni).

In the stomach, no enzymatic activity degrading any of the test substrates was detected (Table 2). As expected, enzymatic activity for starch degradation was higher in the small intestine than in the caecum or colon, and highest in the caecum for pectin, inulin, xylan and cellulose, although the difference between the caecum and the colon was only significant for pectin and xylan (Table 2). However, enzymatic activity for the degradation of pectin, inulin and cellulose was also measured in the small intestine – at values of 54, 36 and 46% of those in the caecum. Only for xylan was the difference much more pronounced, at only 5% of the value in the caecum.

Table 2.
Degradation rate of test carbohydrates by the enzymatic fraction of the gastrointestinal tract contents (μmol of released monosaccharide/g DM of content per minute)

<table>
<thead>
<tr>
<th>GIT section</th>
<th>Test substrate</th>
<th>Starch</th>
<th>Pectin</th>
<th>Inulin</th>
<th>Xylan</th>
<th>Cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Small Int.</td>
<td>77.6 ± 9.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.6 ± 0.3&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.8 ± 0.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>0.8 ± 0.1&lt;sup&gt;A&lt;/sup&gt;</td>
<td>1.8 ± 0.6&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Caecum</td>
<td>40.9 ± 8.2&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.9 ± 1.1&lt;sup&gt;B&lt;/sup&gt;</td>
<td>2.6 ± 0.5&lt;sup&gt;B&lt;/sup&gt;</td>
<td>17.9 ± 5.9&lt;sup&gt;B&lt;/sup&gt;</td>
<td>4.1 ± 1.0&lt;sup&gt;B&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>24.9 ± 28&lt;sup&gt;C&lt;/sup&gt;</td>
<td>2.8 ± 0.7&lt;sup&gt;A&lt;/sup&gt;</td>
<td>2.7 ± 0.3&lt;sup&gt;B&lt;/sup&gt;</td>
<td>10.9 ± 1.1&lt;sup&gt;C&lt;/sup&gt;</td>
<td>3.5 ± 0.6&lt;sup&gt;B&lt;/sup&gt;</td>
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</table>

nd - not detected; within a column, values with different superscripts (A,B,C) differ significantly (RM-ANOVA, Bonferroni)

**DISCUSSION**

The results of this study conform to common knowledge about the distribution of pH and redox potential along the monogastric GI tract: the lowest pH and near-aerobic conditions in the stomach; anaerobic conditions in the intestines; a comparatively high pH in the small intestine; and lower pH in the caecum and colon, indicative of microbial fermentation (Stevens and Hume, 1995; Karasov and Hume,
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In addition, the functional analysis of enzyme activity corresponds, qualitatively, to expectations concerning the presence or absence of enzymatic activity indicative of microbial digestion. However, they also indicate limitations of the methodological approach, in particular in connection with results from similar assays in the sheep GIT (Miltko et al., 2016).

Complex carbohydrates differ in the rate at which they can be digested by microbes (Lavrenčič, 2007). For example, in the case of rabbit caecal contents used as inoculum, in vitro fermentation of cellulose occurs at markedly slower rates than that of pectin or xylan (Marounek et al., 1997), and that of hemicellulose is slower than that of pectin or inulin (Marounek et al., 1997). But even within a group of substances, such as pectins, distinct differences between different substrates can be found (Marounek et al., 1997; Kermauner and Lavrenčič, 2010). These differences might be responsible for our observation that, for example, sugar release from pectin occurred at a lower rate than from xylan.

On the other hand, the diet that donors receive is known to influence the digestion potential of the inoculum taken from them (e.g. reviewed in Hummel et al., 2006). Thus, differences in sugar release rates between inulin and xylan might be explained by a low presence of inulin degrading enzymes in the GIT contents of the nutrias in the present study. In in vitro studies using live microbiomes, instantaneous adaptations to the fermentation substrate are possible, in contrast to the method used here, which only relies on enzymes pre-formed by the microbiome on the diet it was exposed to in vivo.

Regarding quantitative degradation rates, the fact that the small intestine contents were significantly more capable of releasing sugar from starch than the caecum contents, which still released significantly more than the colon contents, is in line with our understanding that starch can be degraded by microbial enzymes, but also that amylase as secreted by the pancreas is predominantly active in the small intestine. When considering the quantitative rates, it should be borne in mind that the residence time of digesta in the various GIT sections will differ dramatically. Whereas total GIT mean retention times of about one day have been reported in the nutria (Hagen et al., 2019), a minority of this time will be spent in the small intestine, with much longer times in the stomach and the large intestine, as found in other mammals (Coombe and Kay, 1965; Wilfart et al., 2007).

The findings of enzymatic activity capable of degrading complex carbohydrates in the contents of the small intestine may be more difficult to explain. Although the values were only about 50% or less of those measured in the caecum, the sheer presence of this enzymatic activity in the small intestine – especially when compared to the clear absence of any activity in the stomach – is surprising. The finding that sugar release rates were always lower in the small intestine starch contents than in the caecum contents in the nutria contrasts with previous findings in sheep (Miltko et al., 2016). In that study, release rates in the small intestine were generally higher than in the caecum (at >500% for, about 120-140% for pectin, inulin and cellulose, and 95% for xylan), even though the ruminant caecum harbours an active microbiome that digests complex carbohydrates (e.g. Welch et al., 2020). Compared to the ruminoreticulum, the sheep small intestine contents achieved release rates of 430% for starch, again corroborating that the small intestine is the main site of amylase production, but also about 90% for pectin and inulin and about 40% for cellulose and xylan. In the sheep, a possible explanation for the lack of a clearer difference between GIT sections could be that enzymes produced upstream of the small intestine by the forestomach
microbiome, and microbes that are in the process of synthesizing these enzymes, are washed downstream in the GIT and will be present in the small intestine contents.

For the nutria, a similar explanation would be theoretically possible, given their digestive strategy that includes coprophagy (see Introduction). However, if the enzyme activity in the small intestine was considered an effect of the presence of microbes in these contents due to coprophagy, we would also expect some of these enzymes in the stomach contents of the same animals. The complete absence of enzymatic activity in the stomach contents, however, speaks against this interpretation. Given our careful approach to dissection, we also consider reflux from the caecum an unlikely explanation for this finding. Thus, one possible explanation is a relatively substrate-unspecific degrading capacity of pancreatic enzymes, which probably does not play an important role in vivo due to the short residence times in the small intestine.

**CONCLUSIONS**

In conclusion, whereas the findings of the present study qualitatively corroborate our understanding of the mammalian digestive tract, the quantitative presence of complex carbohydrate-degrading enzymes in the small intestine of sheep, and in particular of nutrias (present study), raises methodological and biological questions that must be resolved if this method is to be further used to study digestive adaptations in mammals.

**REFERENCES**

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