IMPROVING OF AN *IN VITRO* METHOD TO ESTIMATE DEGRADATION RATE OF SMALL PARTICLES OF STARCH

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ABSTRACT

The aim of this experiment was to improve the washing process during *in vitro* estimation of degradation rate of starch particles. The knowledge of degradation rate of starch particles in the rumen is needed to enhance micro-bacterial protein synthesis and energy supply for dairy cows. The washout of very small particles of starch (M-fraction) in the rumen prevents determination of its degradation rate *in situ*. However, it is possible to measure *in vitro* degradation rate of both a non-washable, potentially degradable fraction (D) and the M fraction.

Centrifugation appeared to be a faster, easier and more reliable method to collect the M fraction of various cereal grains compared to filtration. Medium test bags of 50 cm by 33.3 cm were found to be the most effective for collection of M and D fractions in maize, peas and barley. Results obtained with *in vitro* incubation with amyloglucosidase did not present satisfactory starch degradation curves. Further research will be conducted to improve this technique, or to replace it with an alternative method, such as *in vitro* incubation in rumen fluid.

Key words: starch, degradation, rumen, *in vitro*

Acknowledgments: This study was supported by the Institutional research plan MSM 6215648905 - Biological and technological aspects of sustainability of controlled ecosystems and their adaptability to climate change.

INTRODUCTION

Dairy cows receive most of the energy and protein from microbial fermentation and synthesis in the rumen. With increasing milk production of dairy cows starch has become an important component of dairy diets. Like the other carbohydrates, starch is in the rumen fermented by bacteria to volatile fatty acids with a relatively high proportion of propionic acid. However, part of the starch may escape from rumen fermentation and it is digested and absorbed as glucose in the small intestine with higher efficiency. Particularly for the cows in the early lactation, the diet may contain a higher content of slower degradable starch that prevents acidosis and increases the energy supply to the cow. The information about the extent and site of starch digestion is also important in order to balance energy and nitrogen supply for rumen bacteria to achieve optimal micro-bacterial protein synthesis.

The site and extent of starch degradation in digestive tract is calculated according to degradation rate (kd) and passage rate (kp) of starch fractions (Azarfar et al., 2007; Tamminga et al., 2007). There are three biological methods used to determine degradation rate of feed fractions: *in vivo* (in animals), *in situ* (in place), and *in vitro* (in glass).

A standard technique to estimate degradation rate of feed fractions is an *in situ* incubation of feed samples in small porous nylon bags in the rumen of rumen-fistulated cows. The main feed components such as starch can be sub-divided into following fractions: a washable fraction (W), a non-washable, but potentially degradable fraction (D), and a non-washable, but undegradable fraction (U). The W fraction is the part of feed which is washed away from nylon bags by hand-washing, or by washing in a washing machine. The D fraction and U fraction remain in the bags after washing, however the U fraction is not degradable by rumen microflora (Azarfar et al., 2007; Tamminga et al., 2007). The washable fraction consists of the soluble fraction (S) and of the fraction of small particles (M) which are not soluble in water, but are small enough to be washed away from the nylon bag. This fraction of small particles contains a considerable amount of starch (Reynolds et al., 2002; Tamminga et al., 2007). Although the *in situ* method is the most widely used technique to measure degradation kinetics of feed, it is not possible to determine degradation behaviour of the M fraction *in situ* since this fraction is in the rumen washed away from the nylon bag (Azarfar et al., 2007; Nocek and English, 1986). The rate of degradation and passage rate of this fraction can not be measured and hence these are included in feeding evaluation based on assumptions. It is important to determine degradation rate of the M fraction in order to predict the extent and site of starch degradation more precisely.

Small particles of the M fraction can be collected in water coming out from the washing machine after washing of test bags with feed samples. The M fractions can be then incubated *in vitro* to estimate degradation rate of small particles of starch, simulating the fermentation that occurs in the rumen (*in vivo*).

The aim of this experiment was to find the most suitable modification of the washing process to save labour and material cost, however by getting correct results. Further step was the *in vitro* incubation with enzymes to estimate degradation rate of washable, but not soluble (M) and non-washable, potentially degradable (D) starch fractions from various cereal grains.

MATERIAL AND METHOD

Experiment 1: Improving of the washing process

Nylon bags

Nylon bags (pore size 37) of three different sizes were used and filled with raw material according to Table 1. The big bag was reinforced by a plastic grid to avoid clustering of feed particles. Test bags filled with raw material were washed in the washing machine (Zanker EA 1000, 'wool program', cold water).

Table 1: Different sizes of nylon bags and amount of raw material in mg DM per cm^2

Type of the bag	Size (cm)	Amount of raw material per bag (g)	DM (g)	Surface area (cm^2)	DM (mg/cm^2)
Big bag	50 x 100	200	174	10 000	17
Medium bag	50 x 33,3	67	58	3 330	18
Small bag	24 x 8	6	5	384	14

Raw material

Cereal grains (e.g. peas, maize, barley) and a liquid by-product (LRH47-3, content unknown) were used for this trial. Cereal grains were ground to pass a sieve of 3 mm. Dry matter (DM) content was determined after 4 h of drying in the oven at 103 ºC.

Methods

Basically two different techniques to collect the M fraction after washing were used as follows:

- Filtration
- Centrifugation

Filtration

Filtration took place in a big bucket with inner diameter about 50 cm through two paper filters (double layer) of size 580 by 580 mm. Filters with filtrate were dried in the oven at 70 ºC for 18 h. After drying and weighing the filtrate (M fraction) was collected.

Centrifugation

Water coming out from the washing machine after washing was collected and then centrifuged at 3500 g for 10 minutes. The volume of water used for washing was restricted to 20 L. Approximately 3–5 L of water was used to rinse washing machine after washing and this water was collected as well. After centrifugation the supernatant was pumped away and liquid fraction at the bottom with remaining small particles was centrifuged again in small tubes. Granules at the bottom after centrifugation (M fraction) were collected and dried in the oven at 70 ºC until constant weight (about 24 h).

Calculations

The size of (D+U)-fraction was determined as a weight of raw material remaining in test bags after washing and drying. The size of M fraction was determined either as a weight of material remaining on filters, or as a weight of material obtained by centrifugation after drying. The size of W fraction was calculated as W = 1 – (D + U).

Experiment 2: *In vitro* determination of degradation rate of M fraction and D fraction of starch in maize, barley and peas with enzymes

Principle

The method is based on the *in vitro* incubation of D and M fractions of feed samples with amyloglucosidase hydrolysing starch to glucose. The amount of fermentable starch was measured as the amount of released glucose at time points 30, 60, 120, 180, 240, 300, 360 and 420 minutes. The formed glucose was determined by spectrofotometry with a glucose oxidase reagent. The amount of fermented starch was expressed as the proportion of the total amount of starch determined by enzymatic method.

RESULTS AND DISCUSSION

Effect of the size of nylon bags

Various amount of raw material was weighed into the test bags of different sizes to achieve recommended DM:surface area ratio about 16 mg/cm^2 (see Table 1). After the first trial there was no significant difference in the percentage of material that remained in small, medium and big test bags after washing. While using medium size bags (50 x 33,3 cm) a labour time was reduced. The percentage of raw material that remained in test bags after washing is recorded in Tables 2a (peas), 2b and 2c (maize), and 2d (barley). The big bag was excluded after the first trial, because

preparation of the grid was laborious and also the size of the big bag was not suitable for laboratory equipments (e. g. balance, oven).

Filtration/ Centrifugation

In general, the filtration was the most difficult part of the process. Very fine particles were sticking on filters which depressed their permeability. The speed of filtration differed among used raw materials. In the case of maize the filtration took about 20 h, but filtration of washable fraction of barley took about 4 days and for the pea it was even 7 days. However, the pea was used for the first trial when approximately 400 g of raw material was washed, contrary to next trials when only about 200 g of raw material was washed within one particular trial. If the filtration took several days, the emergence of green and grey spots was observed due to the microbial, or fungal growth on filters. In order to increase speed of filtration the filtration under the vacuum was experienced with peas, but it was also slow procedure, a lot of filters would be needed and problem was how to collect the M fraction from so many small filters. Faster filtration was achieved while churning liquid either by hands, or by electronic mixer. But these improvements had only a pale effect on the speed of filtration. Additional problem was collection of the M fraction from filters after drying, because some particles were joined with a structure of the filter steadily.

Centrifugation was faster and collection of the M fraction was easier compared to filtration. Centrifugation about 23 - 25 L of washable fraction took about 180 min. (9x10 min. of centrifugation + 9x10 minutes for collection of the M fraction).

Table 3 shows percentages of feed fractions after washing procedure with filtration, or with centrifugation to compare its effects on the proportion of feed fractions obtained. In general, higher amount of M fraction was collected by washing process with centrifugation than by washing procedure with filtration. It is possible that some very small particles may pass through filters, but they can be collected by centrifugation. On the other hand, some small particles may be pumped away with supernatant after centrifugation. Since in the case of barley, and especially of peas filtration took several days, the reliability of results may be impacted. Moreover, the quantity and quality of obtained M fraction may be impaired due to the bacterial and fungal growth, which we observed on filters after prolonged filtration. Thus the maize is probably the most favourable raw material for data comparison, because filtration of washable fraction of this cereal did not last as long as filtration of peas and barley. Surprising is a difference in percentages of washable fraction obtained either by washing procedure with filtration, or by washing procedure with centrifugation. The same washing program (‘wool program’) was used for filtration and centrifugation. However the volume of water used for washing was different. For washing process followed by centrifugation the volume of water was restricted to 20 L in order to reduce time needed for centrifugation of washable fraction. Contrary to the expectation that washing in less water may gained smaller amount of washable fraction, the results showed larger proportion of washable fraction and lower proportion of (D + U)-fraction, especially in the case of maize when the size of washable fraction after centrifugation was almost by 25% greater than with filtration. However, in the case of barley percentages of washable fractions obtained were similar.

By the washing process with centrifugation it was possible to collect also M and D fractions of liquid by-product.

Table 2: Percentages of raw material remaining in test bags of different dimensions

Table 2a: Peas

Nylon bag number*	(D+U)-fraction (%)	Deviation from the mean (%)
B1	56,1%	-1,57%
184	62,2%	4,47%
932	54,8%	-2,91%
774	58,6%	0,91%
909	59,8%	2,06%
935	58,6%	0,93%
949	57,1%	-0,58%
M1	56,6%	-1,05%
M3	57,1%	-0,58%
M2	56,0%	-1,67%
Mean	57,7%	0,00%

Table 2b: Maize

Nylon bag number*	(D+U)-fraction (%)	Deviation from the mean (%)
M3	91,1%	3,60 %
M2	85,1%	-2,92%
M1	86,3%	-1,71%
949	86,5%	-1,51%
184	86,8%	-1,25%
774	86,4%	-1,59%
932	90,3%	2,80%
Mean	87,5%	0,00%

Table 2c: Maize

Nylon bag number*	(D+U)-fraction (%)	Deviation from the mean (%)
949	85,6%	-0,55%
184	86,7%	0,55%
774	86,3%	0,16%
932	84,9%	-1,16%
M3	86,4%	0,29%
M1	86,6%	0,52%
M2	86,3%	0,18%
Mean	86,1%	0,00%

Table 2d: Barley

Nylon bag number*	(D+U)-fraction (%)	Deviation from the mean (%)
M1	83,5%	-0,20%
M2	84,0%	0,37%
M3	84,6%	0,97%
949	83,5%	-0,12%
774	83,9%	0,19%
184	83,5%	-0,18%
932	82,6%	-1,02%
Mean	83,7%	0,00%

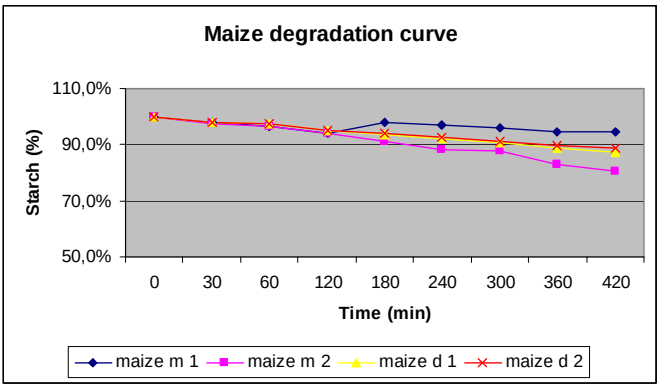
B1 - Big bag; M1, M2, M3 - Medium bags; 184, 932, 774, 909, 935, 949 - Small bags

In vitro estimation of degradation rates of M and D fractions of starch in maize, barley and peas with enzymes

Only results of one run of *in vitro* incubation with amyloglucosidase are available for this report. Therefore the data obtained have more illustrative function and it is impossible to compare them. Temporary results show degradation curves of D and M fractions of maize (Figure 1), peas (Figure 2) and barley (Figure 3). Graph shows percentages of starch remaining at each time point (30, 60, 120, 180, 240, 300, 360 and 420 minutes). The degradation curves obtained show very slow degradation of starch fractions. Normally after 360 min of incubation the starch is almost completely degraded to glucose (Tománková a Homolka, 2004). In this trial even after 420 minutes of incubation there is still more than 80% of starch left. Probably a higher concentration of

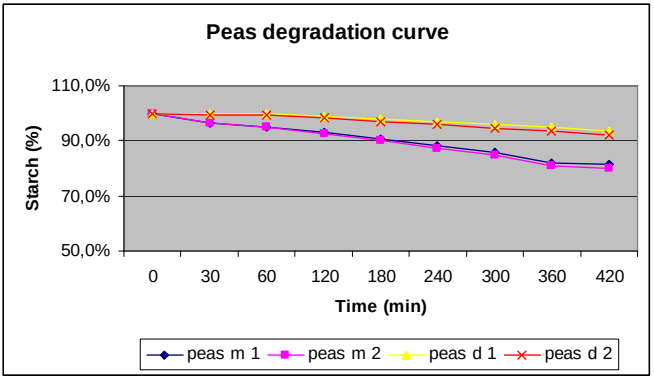
amyloglucosidase should be used. There could also be an influence of the heat (70 ºC) while drying the samples after the washing procedure. But higher temperatures promote starch gelatinization which in a fact should increase the rate of starch degradation to glucose (Hall, 2001; Holm et al., 1998). Degradation of the D fraction was slower than of the M fraction. One fraction of maize (m 1) showed abnormal degradation curve after 120 min of incubation and therefore only the degradation rate of m 2 fraction of maize should be used for further calculations.

Figure 1: Degradation curve of M and D fractions of maize



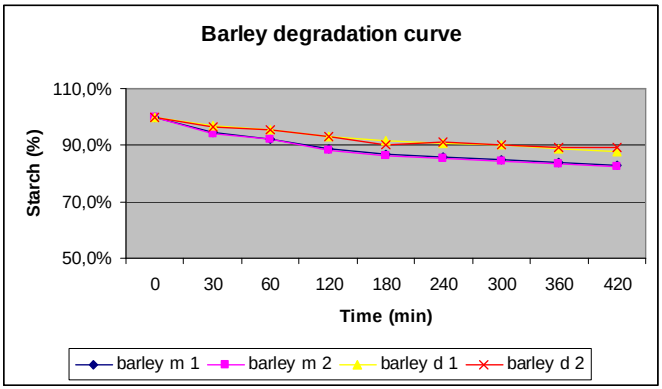
m 1, m 2 = M fractions; d 1, d 2 = D fractions

Figure 2: Degradation curve of M and D fractions of peas



m 1, m 2 = M fractions; d 1, d 2 = D fractions

Figure 3: Degradation curve of M and D fractions of barley



m 1, m 2 = M fractions; d 1, d 2 = D fractions

CONCLUSIONS

It can be concluded that medium test bags of the size 50 x 33.3 cm were suited the best for collection of M and D fractions of various cereal grains.

The partition of fractions gained by washing process with filtration, or with centrifugation is comparable, although there were some differences in percentages of W and M fractions obtained. However, centrifugation appeared to be faster and easier method to collect the M fraction of various cereal grains compared to filtration. In addition, if centrifugation was used, there is no concern about bacterial and fungal growth as it appeared on filters after prolonged filtration. Further examination of washing process should be conducted to confirm reliability of the method and to explain differences between sizes of fractions obtained after washing with filtration, or centrifugation. Also the influence of the volume of water used for washing should be considered in evaluation of washing method.

Results of the *in vitro* incubation with amyloglucosidase did not show satisfactory starch degradation curves. Further research will be conducted to improve this technique, or to replace it with an alternative method, such as *in vitro* incubation in rumen fluid.

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