JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Interactions of α-Amylase and Calcium Chelator during Neutral Detergent Fiber Analysis

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Amylase and calcium chelators, such as disodium ethylene diaminotetraacetate (EDTA), are used in analysis of neutral detergent fiber (NDF) to dissolve starch and pectin, respectively. However, these reagents may interfere with each other's activity. Six combinations of α-amylase and EDTA were examined for determining NDF values of beet pulp (*Beta vulgaris*), ground corn (*Zea mays* L.), timothy hay (*Phleum pratense*), and soybean meal (*Glycine max* L). For treatment A, 2.5 mL of α-amylase was added 5 min after boiling. Other treatments differed as follows: (B) 4.5 mL of α-amylase, (C) 4.5 mL of α-amylase added 30 min after boiling, (D) delayed addition of EDTA to 30 min after boiling, (E) no EDTA, and (F) no α-amylase. Inclusion of EDTA interfered with amylase activity in corn grain samples, and addition of amylase to beet pulp and soybean meal samples reduced the effectiveness of EDTA and increased ash in the NDF residue. Amylase should not be used for samples that do not contain starch. Calculating NDF on an ash-free basis minimized the negative effects of amylase on EDTA activity.

KEYWORDS: Neutral detergent fiber analysis; amylase

INTRODUCTION

Neutral detergent fiber (NDF) represents the cell wall component of plants and is found in most feeds. Although neutral detergent extraction solubilizes some materials that are not digested by mammalian enzymes, NDF can only be slowly fermented by bacteria in the gut of animals and digested (*1*). Van Soest and Wine (*2*) developed the method for measurement of NDF in forages. This method is based on refluxing feed samples in a detergent solution at neutral pH. The detergent solution was comprised of a 3% anionic detergent (sodium lauryl sulfate), which forms soluble complexes with proteins; a chelating agent, disodium ethylene diaminotetraacetate (EDTA), to prevent interference from divalent ions and bind calcium of the calcium−pectin matrix to dissolve pectin; and borate and phosphate as buffers for preventing hydrolysis of hemicelluloses (*3*). Ethylene glycol monoethyl ether was included to prevent foaming but has since been replaced by triethylene glycol, and decahydronaphthalene was initially included but has since been omitted (*1*). Several other modifications of the method have been developed in an effort to apply the procedure to grains and concentrates, as well as to dietary fiber in human foods (*1*). Probably the most common procedural adaptation has been the addition of amylase to the detergent solution. Typically, α-amylase is used for the purpose of removing starches that may cause overestimation of NDF content (*1*). Calcium, required for α-amylase activity, is also added to the NDF solution. A possible complication of the procedure exists because of the potential for EDTA to bind with calcium and thus interfere or prevent α-amylase activity. Conversely, saturation of EDTA by calcium can diminish its chelating effect and thus inadvertently affect NDF results. The purpose of this experiment was to observe the effects of various combinations of α-amylase and EDTA additions on NDF analysis of selected feed ingredients and, in so doing, propose the best practices for different types of feeds.

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MATERIALS AND METHODS

A dry powder form of α-amylase isolated from *Bacillus licheniformis* (Termamyl 60-T) was obtained from Novozymes North America, Inc. (Franklinton, NC). One gram of dry Termamyl 60-T has 60 kilo-Novo units of activity (KNU), where 1 KNU is defined (James Luo, Novozymes, personal communication) as the amount of enzyme needed to degrade 4870 mg of starch per hour under standard conditions (0.3 mM Ca, 37 °C, pH 5.6). A 10% stock solution was prepared by adding 20 g of dry powder to 200 mL of distilled water, stirring for 30 min, and vacuum filtering through Whatman no. 1 filter paper (cat. no. 1001-070; Whatman International Ltd., Maidstone, England) to remove insoluble carrier. The α-amylase stock solution was transferred to a 200-mL Nalgene bottle and stored on ice or at 5 °C until use.

Prior to initiation of fiber analyses, the α-amylase activity was tested to determine the amount of α-amylase required for fiber analysis (*4*) when detergent with or without EDTA is used. Samples (0.5 g) of corn grits (ground to pass a 1-mm screen) were weighed and placed in 600-mL Berzeluis beakers with 100 mL of detergent solution, with or without EDTA, and brought to boil for 5 min. Amylase solution was added to each beaker in 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5 mL aliquots. Each sample was boiled again for 10 min. The beakers were then removed from the fiber apparatus and placed on ice. After solutions were cooled to room temperature (25 °C), 100 μL of 5% Lugol's iodine solution (5% iodine, 10% potassium iodide) was added to each beaker, and after 90 s, the change in color was recorded. Disappearance of the blue iodine/starch reaction color indicates

10.1021/jf0502707 CCC: $30.25
Published on Web 06/18/2005

complete starch hydrolysis. With 2.5 mL (15 KNU) of amylase solution in detergent with no EDTA, most of the color had disappeared within 90 s. When EDTA was included in the detergent solution, 4.5 mL (27 KNU) of amylase solution was needed to reach this same level of activity. Therefore, in the experiment that follows, these two levels of amylase activity were compared.

Four feeds were used as test samples for the experiment: beet pulp (*Beta vulgaris*), ground corn (*Zea mays* L.), timothy hay (*Phleum pratense*), and soybean meal (*Glycine max* L.). Feed processing, solution preparation, and analyses were as described by Mertens (*1*) with the exception that sodium sulfite was omitted from the detergent solution. The analyses were conducted prior to publication of the currently recommended method (*1*), but the differences are not likely to have affected the interactions of interest. In addition to the standard detergent solution, a similar solution was made without EDTA and adjusted to pH 7.0, and 0.25 mol/L EDTA was dissolved in detergent solution and adjusted to pH 7.0.

The feed grains were exposed to six treatment combinations of α-amylase and EDTA. Treatment A used 2.5 mL of α-amylase stock solution (15 KNU) added to 100 mL of detergent solution and 0.5 g of sample at 5 min after boiling on the reflux unit. Other treatments were the same as treatment A with the exceptions noted below. Treatment B used 4.5 mL of α-amylase stock solution (27 KNU). Treatment C used 4.5 mL of α-amylase stock solution added 30 min after boiling on the reflux unit. Treatment D used 2.5 mL of α-amylase stock solution added to 80 mL of NDF solution and an additional 20 mL of 0.05 M EDTA stock solution added 30 min after boiling. Treatment E did not include EDTA. Treatment F did not include an amylase treatment.

Each feed grain was dried overnight in a forced air oven at 55 °C and ground through a 1-mm screen with a Wiley mill, and 0.5 g samples were weighed into numbered 600-mL beakers. The buffer combinations as outlined above (A−F) were added to the beakers, and each sample was refluxed at boiling temperatures for 60 min (*1*). To enhance the filtering of samples containing significant levels of starch, several precautionary steps were taken to avoid clogging of the crucibles. Gooch crucibles were kept hot (in the oven or in boiling water) until filtering began. In addition, care was taken not to let the crucibles dry completely during filtering. After refluxing was complete, each sample was slowly poured into Gooch crucibles containing boiling water. Each sample was carefully filtered 3 times with boiling water (20 mL), then twice with acetone (20 mL) in a well-ventilated hood. Residual acetone was allowed to evaporate, and samples were placed in a forced-air oven for at least 8 h at 105 °C. Subsequently, each sample was weighed hot after equilibrating for 10 s, and NDF values were calculated. After NDF was measured on 10 replicates per feed and treatment, detergent-insoluble ash was determined on five replicates, and detergent-insoluble nitrogen (N) was determined on the other five. The NDF residues were ashed overnight in a muffle furnace at 500 °C. Ash weights were recorded after tempering at 105 °C for 3 h. Percentage N for five NDF residues was determined by Dumas (*4*) combustion analysis (Leco N analyzer, model no. FP-428; Leco Corporation, St. Joseph, MI). Crude protein was determined by multiplying N results by 6.25. All results are expressed as a percentage of dry sample weight.

Statistical Analysis. The experiment was analyzed as a completely randomized design with 10 replications of each treatment per feed sample for NDF determination and 5 replications of each feed for detergent-insoluble ash and detergent-insoluble N. All statistical analyses were carried out using the statistical software JMP (Ver. 5; SAS Inc., Cary, NC). Mean separation was determined using Student's *t* test, ($P < 0.05$) for each feedstuff when ANOVA was significant.

RESULTS AND DISCUSSION

It has been reported in previous experiments (*1*) that certain samples (i.e. those containing high levels of starch) can be difficult to filter, and NDF results may be affected by filtering difficulties. In general, there were no difficulties associated with filtering in this experiment. When our filtering method was used, all samples, including those high in starch, pectin, or both filtered rapidly (<5 min). The four feed grains used in this study were selected as samples that contained high levels of fiber (timothy hay), starch (corn grain), protein (soybean meal), and pectin (beet pulp and soybean meal).

Table 1. Neutral Detergent Fiber (Percent of Dry Matter) of Selected Feedstuffs after Various Amylase and EDTA Treatments during Fiber Analysis[a,b]

treatment name	A	B	C	D	E	F	SE
amylase amount (mL)[c]	2.5	4.5	4.5	2.5	2.5	0	
time[d] of amylase addition	5	5	30	5	5		
EDTA final concn (M)	0.05	0.05	0.05	0.05	0	0.05	
time[d] of EDTA addition	0	0	0	30		0	
feed							
beet pulp	47.5 a	48.2 ab	47.5 a	49.2 b	50.8 c	47.3 a	0.37
corn grain	15.8 c	14.9 b	13.6 a	13.1 a	13.5 a	15.0 bc	0.26
soybean meal	10.0 ab	10.5 b	9.6 a	9.8 a	17.0 c	9.8 a	0.18
timothy hay	64.2 b	64.3 b	63.7 a	64.5 b	65.0 c	64.4 b	0.16

[a] Means ($n = 10$) across rows followed by different letters are significantly different (Student *t* test; $P < 0.05$). [b] Detergent solution (*1*) did not include sodium sulfite; samples were not corrected for ash. [c] Amylase stock solution = 10% Termamyl 60-T (Novozymes North America, Inc., Franklinton, NC) in detergent solution; added to 100 mL of detergent solution and 0.5 g of sample. [d] Time (min) of amylase or EDTA addition after reflux boiling began; total boil time = 60 min.

Ideally, the NDF residues obtained after refluxing in neutral detergent solution should include all celluloses, hemicelluloses, and lignin in the sample (*6*). In addition, some proteins and minerals also remain. McQueen and Nicholson (*6*) observed that there is no standard feedstuff for comparing NDF determination in feeds. Therefore, criteria for selecting the best procedure for NDF analysis need to be established. In the present study, effectiveness of EDTA was evaluated by its ability to remove calcium and pectin and thereby decrease NDF, detergent-insoluble ash residue, and ash-free NDF from beet pulp and soybean meal. The effectiveness of amylase was evaluated for its ability to decrease NDF of corn grain. Timothy hay was included to ensure that treatments did not affect dissolution of cellulose, hemicellulose, or lignin.

Percentage NDF results are shown in **Table 1**. Treatment C (adding the greater amount of amylase 30 min after boiling began) yielded the lowest NDF for the four feed grains tested. Robertson and Van Soest (*7*) reported that EDTA may inhibit α-amylase activity. Thus, treatment E was included using detergent solution without EDTA. The NDF was highest when there was no EDTA present in treatment E in three of four of the feed grains tested, the exception being corn grain. While EDTA was essential for three of four feeds, it did appear to decrease amylase activity on corn grain, the feed in this experiment with the greatest starch content. However, either adding amylase after refluxing for 30 min or delaying the addition of EDTA appeared to resolve this problem.

Ash in NDF residues is shown in **Table 2**. For beet pulp and soybean meal, omission of EDTA resulted in greater ash in the NDF residue, presumably because EDTA did not dissolve divalent cations such as Ca^{2+} and Mg^{2+}. Soybean meal is about 6% ash and 2% divalent elements (*8*), so the large differences in ash solubility were surprising. However, the amylase solution also contained large quantities of Ca^{2+}, which may have precipitated. In contrast, removing amylase from detergent solution decreased ash in the NDF residue for beet pulp and soybean meal, suggesting that amylase inhibited EDTA effectiveness.

Table 2. Neutral Detergent-Insoluble Ash (Percent of Dry Matter) of Selected Feedstuffs after Various Amylase and EDTA Treatments during Fiber Analysis[a,b]

treatment name	A	B	C	D	E	F	SE
amylase amount (mL)[c]	2.5	4.5	4.5	2.5	2.5	0	
time[d] of amylase addition	5	5	30	5	5		
EDTA final concn (M)	0.05	0.05	0.05	0.05	0	0.05	
time of EDTA addition	0	0	0	30		0	
feed							
beet pulp	7.1 a	8.4 b	8.1 b	9.0 c	13.0 d	7.5 ab	0.32
corn grain	4.5 d	1.5 c	–1.4 a	0.3 bc	1.7 c	–0.1 ab	0.53
soybean meal	10.6 c	10.7 c	4.9 a	6.8 ab	9.1 bc	8.0 ab	1.2
timothy hay	1.4	1.1	1.3	1.4	1.0	1.4	0.20

[a] Means ($n = 5$) across rows followed by different letters are significantly different (Student t test; $P < 0.05$). [b] Detergent solution (*1*) did not include sodium sulfite. [c] Amylase stock solution = 10% Termamyl 60-T (Novozymes North America, Inc., Franklinton, NC) in detergent solution; added to 100 mL of detergent solution and 0.5 g of sample. [d] Time (min) of amylase or EDTA addition after reflux boiling began; total boil time = 60 min.

Table 3. Ash-Free Neutral Detergent Fiber (Percent of Dry Matter) of Selected Feedstuffs after Various Amylase and EDTA Treatments during Fiber Analysis[a,b]

treatment name	A	B	C	D	E	F	SE
amylase amount (mL)[c]	2.5	4.5	4.5	2.5	2.5	0	
time[d] of amylase addition	5	5	30	5	5		
EDTA final concn (M)	0.05	0.05	0.05	0.05	0	0.05	
time[d] of EDTA addition	0	0	0	30		0	
feed							
beet pulp	39.7 b	40.4 b	40.3 b	39.8 b	38.3 a	39.9 b	0.40
corn grain	12.3 a	13.1 a	15.1 b	12.5 a	12.0 a	15.6 b	0.59
soybean meal	–0.40 a	0.17 ab	4.7 cd	3.1 bc	7.7 d	1.9 ab	1.17
timothy hay	62.3 a	63.2 a	62.6 a	63.0 a	64.1 b	62.4 a	0.29

[a] Means ($n = 5$) across rows followed by different letters are significantly different (Student t test; $P < 0.05$). [b] Detergent solution (*1*) did not include sodium sulfite. [c] Amylase stock solution = 10% Termamyl 60-T (Novozymes North America, Inc., Franklinton, NC) in detergent solution; added to 100 mL of detergent solution and 0.5 g of sample. [d] Time (min) of amylase or EDTA addition after reflux boiling began; total boil time = 60 min.

Ash-free NDF results are shown in **Table 3**. Although some differences among treatments were still significant, the magnitudes of differences were reduced substantially. Omission of amylase, or adding it after refluxing for 30 min, increased ash-free NDF residue of corn grain, probably because starch was not completely removed. Omission of EDTA, or adding EDTA or amylase 30 min after refluxing, increased ash-free NDF in soybean meal probably because solubility of pectin was reduced. Surprisingly, omission of EDTA actually decreased ash-free NDF from beet pulp 1.4 percentage units (treatments A vs E) even though ash content increased. We have no explanation as to why. Expressing results on an ash-free basis appeared to eliminate much of the effect of reduced EDTA activity for beet pulp but to a lesser extent for soybean meal.

Crude protein in NDF residues is shown in **Table 4**. As expected, treatments did not affect these results to a very large degree. Crude protein may have been affected by the omission of sodium sulfite from the detergent solution (*1*).

Table 4. Neutral Detergent-Insoluble Crude Protein (Percent of Dry Matter) of Selected Feedstuffs after Various Amylase and EDTA Treatments during Fiber Analysis[a,b]

treatment name	A	B	C	D	E	F	SE
amylase amount (mL)[c]	2.5	4.5	4.5	2.5	2.5	0	
time[d] of amylase addition	5	5	30	5	5		
EDTA final concn (M)	0.05	0.05	0.05	0.05	0	0.05	
time[d] of EDTA addition	0	0	0	30		0	
feed							
beet pulp	6.6 ab	7.0 b	6.2 a	7.0 b	6.2 a	6.8 b	0.17
corn grain	1.3 b	1.4 b	1.2 b	1.3 b	1.0 a	1.2 b	0.06
soybean meal	1.1 a	1.3 a	1.2 a	1.3 a	2.2 b	1.2 a	0.06
timothy hay	3.2 b	3.1 a	3.1 a	2.9 a	2.9 a	2.9 a	0.08

[a] Means ($n = 5$) across rows followed by different letters are significantly different (Student t test; $P < 0.05$). [b] Detergent solution (*1*) did not include sodium sulfite. [c] Amylase stock solution = 10% Termamyl 60-T (Novozymes North America, Inc., Franklinton, NC) in detergent solution; added to 100 mL of detergent solution and 0.5 g of sample. [d] Time (min) of amylase or EDTA addition after reflux boiling began; total boil time = 60 min.

Inclusion of EDTA decreased amylase activity in corn grain samples, and addition of amylase to feed samples reduced effectiveness of EDTA and increased ash in the NDF residue. We recommend using amylase only for feedstuffs high in starch (e.g., corn grain) and in amounts that are great enough to compensate for competition for calcium with EDTA. Feeds that are high in insoluble pectin (e.g., soybean meal) should not be treated with amylase. In any case, calculating NDF on an ash-free basis minimized the effects of low EDTA activity in the presence of amylase.

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Received for review February 4, 2005. Revised manuscript received April 26, 2005. Accepted May 17, 2005.

JF0502707