



Research article

Properties of corn-expressed carbohydrase AC1 in swine diets and its effects on apparent ileal digestibility, performance, hematology, and serum chemistry

Philip A. Lessard, Xuemei Li, Jonathan N. Broomhead¹, Matthew H. Parker², Christopher Bailey³, R. Michael Raab^{*}

Agrivida, Inc., 78E Olympia Avenue, Woburn, MA, USA

ARTICLE INFO

Keywords:

Carbohydrase
Beta-glucanase
Animal nutrition
Swine
Feed additive

ABSTRACT

Carbohydrases are often incorporated into livestock feed as digestive aids to improve animal performance. AC1 is a thermostable carbohydrase with β -1,4-glucanase, endo-cellulase, and cellobiohydrolase activity. AC1 has been expressed in corn, where it accumulates in the grain for easy inclusion in animal diets. Incorporating the enzyme in high-fiber diets (corn-soy supplemented with distiller's dry grains with solubles) that were fed to 5-week-old pigs led to a trend of decreasing viscosity of the digesta as the dose of the enzyme increased ($P = 0.092$). AC1 also tended to increase the apparent ileal digestibility (AID) of neutral detergent fiber ($P = 0.076$). When fed diets containing 2126 U/kg AC1, pigs experienced no adverse effects in terms of performance metrics (body weights, average daily gain, average daily feed intake and gain-to-feed ratio), hematology, blood chemistry or general health when compared to pigs fed a control diet that lacked AC1.

1. Introduction

Carbohydrases have been used extensively in diets for swine and poultry (Adeola and Cowieson 2014). Carbohydrases encompass a broad class of enzymes that include xylanases, glucanases, amylases, glucosidases, cellulases, galactosidases, and pectinases, among others. Carbohydrases improve animal nutrition and health by hydrolyzing polysaccharides and reducing the viscosity of the digesta, which allows digestive enzymes to circulate more freely and could potentially improve the absorption of nutrients, while also promoting beneficial changes in gastrointestinal microbiota (Kiarie et al. 2017). Non-starch polysaccharides (NSPs) in particular have anti-nutritive properties that can be reduced by carbohydrases. Solubilization of NSPs can improve microbial fermentation in the hindgut, and exogenous carbohydrases can complement digestive enzymes that may be limiting, especially in young animals (Li et al., 2018; Swiatkiewicz et al., 2016; Tsai et al., 2017).

Bacterial or fungal cultures have historically been widely used to produce feed enzymes for animal diets. However, since corn is a major feed component for livestock, using corn to produce feed enzymes

presents a convenient option for incorporating additives such as enzymes into animal feed. AC1 is a carbohydrase with sequence similarity to a β -1,4-glucanase from the thermophilic marine bacterium *Thermatoga maritima*. AC1 has been produced in corn grain, and previous work has shown that corn-expressed AC1 is both safe and effective in poultry diets (Broomhead et al. 2019). Furthermore, initial results have shown that this carbohydrase has value in swine diets (work in progress). Here we assess the properties of corn-expressed AC1, its role in digestion, and the ability of swine to tolerate high inclusion rates of this enzyme in typical diets.

2. Materials and methods

2.1. Purification of AC1

AC1 grain was milled to a fine meal in a Retsch SM100 cutting mill with a 1mm screen. One hundred grams of corn meal was mixed with 500 mL of 20mM sodium acetate, 1mM EDTA, pH 4.0 buffer and stirred overnight at 4 °C. After centrifugation at 15,000 × g for 30 min, the

* Corresponding author.

E-mail address: michael.raab@agrivida.com (R.M. Raab).¹ Current address: Perstorp US, Glencoe, MO, USA.² Current address: Versatope Therapeutics, Lowell, MA, USA.³ Current address: Department of Poultry Science, Texas A&M University, College Station, TX.

supernatant was transferred to a 500 mL centrifuge bottle and placed into an 80 °C water bath for 30 min. After chilling on ice for five minutes, the suspension was centrifuged at 15,000 × g for 30 min and then decanted into a 500 mL graduated cylinder. Approximately 450 mL of supernatant was collected and combined with 225 mL of 20 mM sodium acetate, 1mM EDTA, 3 M ammonium sulfate, pH 4.0 buffer, and the mixture was stirred on ice for 20 min. The solution was filtered through a 0.45 µm vacuum filter. The sample was loaded onto a 20 mL HiTrap Phenyl FF high substitution column and eluted with a buffer containing 20mM Tris-HCl, 1mM EDTA, pH8. The fractions containing AC1 enzyme with minimal amount of protein impurities (as determined via polyacrylamide gel analysis) were pooled. The pool was concentrated to under 5 mL using centrifugal concentrators. The concentrate was exchanged into 20 mM Tris-HCl, 1 mM EDTA, pH 8 using two PD-10 desalting columns. The solution was diluted to 50 mL using 20 mM Tris-HCl, 1 mM EDTA, pH 8.0 buffer, and then was loaded onto a 1 mL MonoQ column and eluted in a buffer containing 20mM Tris-HCl, 1mM EDTA, 1M NaCl, pH8.0. The fractions containing AC1 enzyme activity with minimal impurities were pooled and concentrated to approximately 1 mL using centrifugal concentrators as described above. The concentrated pool was further purified on a 120 mL Sephacryl S100 gel filtration column and eluted with a buffer containing 100mM MES, 300mM NaCl, pH 6.3. The fractions containing AC1 enzyme activities with minimal impurities were pooled and concentrated to approximately 0.64 mL, and this pool was mixed with 1 volume of 80 % glycerol before storage at -20 °C. To measure the AC1 protein concentration, the protein was diluted by a factor of 10 in 50mM MES, 150mM sodium chloride, 40 % glycerol, pH 6.3 buffer, and absorbance at 280 nm (A280) was measured in triplicate. The protein concentration was calculated by dividing A280 by the AC1 protein extinction coefficient (2.59 mL/mg) and multiplying by the dilution factor. Purity of the preparation was evaluated by SDS-PAGE and found to be >95 %.

2.2. AC1 assays

AC1 activity in grain or feed samples was carried out as described previously (Ayres et al., 2018; Jasek et al., 2018). In this context, one unit (U) of AC1 activity equals 1 µmol/min glucose reducing equivalents released from 1 % barley-β-glucan at 80 °C, pH 6.5. For tests of thermal stability, 400 µL of diluted protein from crude extracts, purified protein or a commercial β-glucanase (Sigma, catalog #G8673) were placed in a shaking incubator at temperatures of 25, 50, 60, 70, 80, 90, 95 and 100 °C. Incubation at each temperature was carried out for 5 min with shaking at 1000 rpm. The temperature of sample wells was checked using a Dual Channel Digital Thermometer (Fisher Scientific). Samples were then cooled to room temperature before being used in the Beta-Glucosidase assay described above, except that the enzyme/substrate mixture was incubated at 25 °C instead of 80 °C. Data from the thermal stability measurements were tested using a univariate analysis of variance to evaluate the effects of enzyme and temperature with a significance threshold set at $P = 0.05$. Contrast between the extracted enzymes and either the purified enzyme or the commercial enzyme were made using a oneway analysis of variance with a significance threshold set at $P = 0.05$.

2.3. Assays for additional enzyme activity

Additional enzymatic activities of AC1 were tested by incubating the protein in the presence of various diagnostic substrates. In each case, a positive control enzyme was tested simultaneously to demonstrate that the chosen substrate was appropriate for detecting the type of activity in question. All reactions used 5 nM purified AC1, or 5 µL of control enzyme at the indicated concentrations (see below), in 200 mM sodium phosphate, 0.01 % (v/v) Tween 20, pH 6.5. Reactions were carried out in triplicate for both AC1 and control enzymes, and blank reactions with no enzyme were performed in duplicate. All reactions were performed in 1.5 mL Eppendorf tubes for one hour at both 37° and 80 °C. Absorbance

values for the average of the two blanks were subtracted from the values for each enzymatic reaction and activities are reported in units of net increases in absorbance at the wavelengths indicated.

β-1,4 -glucanase assays: Each assay contained one tablet of Beta-Glucosidase substrate (AZCL-barley-β-glucan; Megazyme, Wicklow, Ireland; catalog #TBGZ-1000T). The control enzyme was 100 mg/mL β-1,4-glucanase (Sigma G8673). Reactions were terminated by the addition of 1 mL of 2 % Tris base. Samples were centrifuged for 10 min at 15,000 × g, 20 µL of supernatant was added to 100 µL of water in a microplate, and the absorbance at 590 nm was recorded.

Endocellulase assays: Each assay contained one tablet of Cellazyme C substrate (AZCL-HE-cellulose; Megazyme, catalog #T-CCZ) in 500 µL buffer. The control enzyme was 100 mg/mL *Aspergillus niger* cellulase (Sigma catalog # 22178). Reactions were terminated by the addition of 1 mL of 2 % Tris base. Samples were centrifuged for 10 min at 15,000 × g, 5 µL of supernatant was added to 100 µL of water in a microplate, and the absorbance at 590 nm was recorded.

Cellobiohydrolase (exocellulase) assays: The substrate was 1 mM pNP-D-cellobioside (Sigma catalog #N5759) and the positive control enzyme was CBHI from *Trichoderma longibrachiatum* (Megazyme catalog #E-CBHI; 0.5 Units/µL). Reaction volumes were 100 µL; reactions were terminated by the addition of 100 µL of 2 % (w/v) Tris base. Aliquots of 150 µL were transferred to a microplate and the absorbance at 405 nm was recorded.

Endo-β-1,3-glucanase assays: Each assay contained one tablet of 1,3-β-glucosidase HS substrate (AZCL-curdan; Megazyme catalog #ET-CUR200) and the positive control enzyme was *Trichoderma* sp. 1,3-β-D-glucanase (Megazyme catalog #E-LAMSE; 50 Units/mL). Reactions were carried out as described above for the endocellulase assays.

Beta-xylosidase assays: The substrate was 1 mM pNP-β-D-xylopyranoside (Sigma catalog #N2132) and the control enzyme was a β-xylosidase from *Aspergillus niger* (Sigma catalog #X3501). Reaction volumes were 100 µL; reactions were terminated by the addition of 100 µL of 2 % (w/v) Tris base. Aliquots of 150 µL were transferred to a microplate and the absorbance at 405 nm was recorded.

2.4. Feeding trial 1

The first feeding trial was conducted at North Carolina State University (Raleigh, NC), and all animal experimental procedures were approved by the North Carolina State University Animal Care and Use Committee. Sixty pigs (10.2 ± 1.3 kg) at 5 wk of age were individually housed and blocked based on initial body weight and sex. Within each block, pigs were randomly allotted to 6 dietary treatments based on a 2 × 3 factorial arrangement. The first factor was distiller's dried grains with solubles (DDGS) inclusion (15 or 30 %), and the second factor was AC1 supplementation (0, 150, or 450 U/kg feed). The source of AC1 was ground GraInzyme® AC1 corn with 50 U/g of β-glucanase activity. Two different basal diets (99.1 %) were mixed depending on the inclusion of DDGS, and then AC1 corn was added at the rates of 0 %, 0.3 %, and 0.9 % premixed with normal corn to reach the target glucanase activities of 0, 150, and 450 U/kg feed in each dietary treatment, respectively. The diet composition was summarized in Table 1, and nutrient levels of all diets met the requirement suggested by NRC (NRC 2012). Feed intake was recorded at weekly intervals during the trial.

The experimental period was 21 d. Pens (1.50 × 0.74 m) with slatted floors were equipped with 1 nipple drinker and 1 self-feeder. Pigs had free access to water and feed. On d 14, titanium dioxide (0.3 %) was blended into experimental diets as an indigestible marker to measure AID. On d 21, all pigs were euthanized via captive-bolt stunning. Digesta (40 mL) was collected from the ileum (30 cm of small intestine from the ileo-cecal junction) to measure viscosity immediately after euthanasia. Ileal digesta (100 mL) was collected and frozen at -20 °C.

The method to measure digesta viscosity was described by (Passos et al., 2015) with a viscometer (Brookfield Digital Viscometer, Model DV2TLV, Brookfield Engineering Laboratories Inc., Stoughton, MA). The

Table 1. Composition of diets for feeding trial 1.

DDGS, %	15			30		
	0	150	450	0	150	450
Glucanase, U/kg						
Ingredient, %						
Yellow corn	49.765	49.465	48.865	37.955	37.655	37.055
Poultry fat	2.00	2.00	2.00	2.00	2.00	2.00
DDGS	15.00	15.00	15.00	30.00	30.00	30.00
SBM	23.00	23.00	23.00	20.00	20.00	20.00
Whey permeate	5.00	5.00	5.00	5.00	5.00	5.00
Blood plasma	2.00	2.00	2.00	2.00	2.00	2.00
Supplement	0.00	0.30	0.90	0.00	0.30	0.90
L-Lys HCl	0.43	0.43	0.43	0.45	0.45	0.45
DL-Met	0.10	0.10	0.10	0.05	0.05	0.05
L-Thr	0.08	0.08	0.08	0.05	0.05	0.05
Salt	0.22	0.22	0.22	0.22	0.22	0.22
Vitamin premix	0.03	0.03	0.03	0.03	0.03	0.03
Mineral premix	0.15	0.15	0.15	0.15	0.15	0.15
Dical Phosphate	0.55	0.55	0.55	0.32	0.32	0.32
Limestone	1.30	1.30	1.30	1.40	1.40	1.40
Zinc oxide	0.25	0.25	0.25	0.25	0.25	0.25
Mecadox 10	0.125	0.125	0.125	0.125	0.125	0.125
Total	100.00	100.00	100.00	100.00	100.00	100.00
Composition¹						
ME, kcal/kg	3386	3386	3386	3394	3394	3394
Lys, %	1.24	1.24	1.24	1.24	1.24	1.24
Met + Cys, %	0.7	0.7	0.7	0.7	0.7	0.7
Trp, %	0.21	0.21	0.21	0.21	0.21	0.21
Thr, %	0.73	0.73	0.73	0.73	0.73	0.73
Ca, %	0.71	0.71	0.71	0.7	0.7	0.7
Available P, %	0.33	0.33	0.33	0.33	0.33	0.33
DM, %	88.98	89.05	89.31	88.88	89.2	89
NDF, %	13.95	13.93	12.53	18.6	17.65	19.16
ADF, %	3.94	3.88	3.71	5.26	5.06	5.26
AC1 Activity (U/kg)	2	159	554	13	158	561

¹ ME, Ca, available P: calculated values; DM, NDF, ADF and AC1 β -1,4 -glucanase activity: analyzed values.

digesta samples were centrifuged at $3,000 \times g$ for 5 min and then the supernatant was pipetted out to a 2 mL tube and centrifuged at $12,500 \times g$ for 5 min. Viscometer was set at 25 °C, and 0.5 mL of digesta supernatant was placed in the viscometer. The final result was calculated as the average of viscosity at 45.0 s^{-1} and 22.5 s^{-1} shear rates.

Before analysis, ileal digesta was freeze-dried (24D x 48, Virtis, Gardiner, NY). Feed and ileal digesta samples were ground and analyzed for dry matter (DM; AOAC Method 934.01). Titanium concentration was measured according to the previously described protocol (Myers et al., 2004). The gross energy (GE) was determined using a calorimeter (Model 6200, Parr Instrument Company). Crude fat was measured using a modified ether extract method (AOAC Method 920.39). Nitrogen in the feed and digesta samples was measured using a TruSpec N Nitrogen Determinator (LECO Corp., St. Joseph, MI) to calculate crude protein (CP; $6.25 \times N$). Samples of feed and ileal digesta were analyzed sequentially for neutral detergent fiber (NDF) and acid detergent fiber (ADF) using the method of (Van Soest et al., 1991) in a batch processor (Ankom Technology Corp, Fairport, NY). Apparent ileal digestibility of DM, GE, Crude fat, CP, NDF, and ADF were calculated as a function of the titanium concentration in the feed and digesta with Eq. (1):

$$\text{AID, \%} = \left(1 - \frac{\text{Ti}_{\text{feed}} \times \text{Nutr}_{\text{digesta}}}{\text{Ti}_{\text{digesta}} \times \text{Nutr}_{\text{feed}}}\right) \times 100\%, \quad (1)$$

where Ti_{feed} represents the titanium concentration in the feed, $\text{Ti}_{\text{digesta}}$ is the titanium concentration in the ileal digesta, $\text{Nutr}_{\text{feed}}$ represents the

nutrient concentration in the feed, and $\text{Nutr}_{\text{digesta}}$ is the nutrient concentration in the ileal digesta.

2.5. Feeding trial 2

The second feeding trial was conducted at RTI, LLC (Brookings SD), and all animal experimental procedures were approved by the Institutional Animal Care and Use Committee at RTI. The trial was a randomized complete block and double-blinded design in which 40 animals were ranked by weight and blocked by sex, then randomly assigned to two treatment groups (negative control or carbohydrase treatment), each treatment group containing 20 pigs with males and females equally represented. Once assigned to treatment group, the pigs in each treatment group were ranked by weight and sequentially divided into 10 sets of two pigs each of the same sex to ensure relative uniformity of weight of the two pigs in each pen. Each set of two pigs was then randomly assigned to one of 20 pens.

Pigs were fed nutritionally complete corn-soy-DDGS diets in mash form, manufactured by Millbrook Feeds, LLC (Mitchell SD; Table 2). The two diets were identical except that 12.6 kg of the corn in the treated diet was replaced by 12.6 kg of corn expressing AC1 (170 U/g of β -1,4-glucanase activity) per tonne of feed, which provided the treated diet with approximately 2126 U/kg AC1 activity, based on analysis of the completed feed. Pigs had free access to water and feed. Pigs were observed at least daily for general health from Study Days 0–42. Pigs were individually weighed on Study Days 0, 21, and 42. Feed

Table 2. Composition of basal diet for the second feeding trial.

Ingredients	Control	AC1
Corn	45.87	44.61
Corn expressing AC1		1.26
Soymeal Full Fat	30.896	30.896
Distillers HI Pro 38 %	10	10
Soybean Meal 47 %	7.654	7.654
Veg Oil	1.891	1.891
Super Cal 38 Gilmore	1.435	1.435
Dical 21 Biofos	1.039	1.039
Salt Mixing	0.519	0.519
Choline Chloride 60 %	0.208	0.208
MB Turkey Vtm Pmx	0.2	0.2
Biolys	0.18	0.18
StreessesPlus	0.048	0.048
Magnesium Oxide	0.047	0.047
Vitamin E 50 % 227	0.01	0.01
Copper Sulfate 25.2 %	0.003	0.003
Total	100	100
Composition	Common basal diet	
ME, kcal/kg	3402	
Lys, %	1.13	
Met + Cys, %	0.63	
Trp, %	0.25	
Thr, %	0.75	
Ca, %	0.84	
Available P, %	0.37	
DM, %	90.79	
NDF, %	13.8	
ADF, %	4.9	
AC1 activity (U/kg)*	2126	

* β -1,4-glucanase activity, detected only in diet B.

consumption was recorded throughout the study and total feed consumed per pen was calculated on Study Days 21 and 42.

Two samples of blood were collected from the jugular vein of each pig on Study Days 0, 21, and 42. For the first sample, approximately 6 mL of whole blood was collected into one serum separation tube for each pig. Serum was submitted to the Iowa State University Clinical Pathology Laboratory (ISU CPL) for serum chemistry analysis, including albumin, glucose, phosphorus, alanine transaminase (ALT), and creatine phosphokinase (CK). For the second sample, approximately 5 mL of whole blood was collected into one tube containing EDTA for each pig. Immediately after collection, tubes were gently inverted several times to ensure thorough mixing of the blood and anticoagulant. Samples were submitted on ice packs within 24 h of collection to ISU CPL for hematology analysis including total white blood cells count (WBC), WBC differential (i.e., neutrophil, lymphocyte, monocyte, eosinophil, basophil), absolute large unstained cells (LUC), red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelet count, and mean platelet volume (MPV). These tests include several markers that are sensitive to malabsorption of nutrients such as carbohydrates, minerals and vitamins, kidney and liver function, disease, infection, stress, hydration, and overall health status (Cooper et al., 2014; Onasanya et al., 2015). All pigs were euthanized and necropsied on Study Day 42. Pigs were examined by a board-certified pathologist for visual abnormalities or indications of toxicity in the major organ systems.

Data were analyzed using IBM Corp. IBM SPSS Statistics for Windows, Version 25.0 (Armonk, NY: IBM Corp.SPSS Statistical Software). Pen was used as experimental unit for each analyzed variable, except for

hematology and blood chemistry data, in which the pig providing the sample served as the experimental unit. All production data were initially evaluated for both Normality and Homogeneity of Variance by Treatment. The ANOVA model included treatment, block, and their interactions, and P -values < 0.05 were considered significant in all comparisons. Any reference to trends assumes P -values < 0.10 .

3. Results

3.1. Properties of AC1

The thermal tolerance of the AC1 was tested over a range of temperatures. AC1 activity was tested in crude extracts from three representative AC1 crops (Lot numbers AV_AC1_0070, AV_AC1_0075, and AV_AC1_0077), AC1 that had been column-purified from AC1-expressing grain, and a commercially available thermostable β -glucanase (Sigma, catalog #G8673). All AC1 samples and the commercial β -glucanase samples were diluted using AC1 extraction buffer (Ayres et al., 2018). The relative glucanase activity in each of the AC1 product batches and AC1 purified from AC1-expressing grain at the different temperatures was determined and are presented in Figure 1. Analysis of variance among the three extracted enzymes as well as the purified enzyme and the commercial enzyme revealed significant differences between enzymes ($P = 0.009$) and between temperatures ($P < 0.001$). The AC1 in crude aqueous extracts from three representative product batches demonstrated 100 % activity after 5 min incubation at temperatures from 50 - 100 °C relative to their activity at 25 °C. The AC1 that had been purified from grain maintained more than 82 % activity between 60 °C to 100 °C. The commercial thermostable β -glucanase maintained 100 % activity at 70 °C relative to its activity measured at 25 °C, but retained only 25.9 % activity at 80 °C, and then lost activity completely at temperatures of 90 °C and above (Figure 1). Pairwise one-way ANOVA between each of the extracted enzymes and either the purified enzyme or the commercial enzyme revealed significant differences ($P < 0.001$ in each case).

3.2. Enzymatic activities of AC1

AC1 protein purified from extracts of the representative AC1 product batch AV_AC1_0075 was tested for the presence of other measurable enzymatic activities. The enzymatic activities that were tested included β -1,4-glucanase, β -1,3-glucanase, β -xylosidase, cellobiohydrolase, and endocellulase. The detectible enzymatic activities of the AC1 and a positive control enzyme with the corresponding activity (obtained from commercial sources) were compared for each enzymatic assay tested. The results show that the primary activity of AC1 protein is β -1,4-glucanase. AC1 also possesses easily-detected endocellulase and cellobiohydrolase activities in addition to low but detectable levels of β -1,3-glucanase activities (Table 3). Beta-xylosidase activity of AC1 was positive but only slightly above the blank control. In similar assays, no measurable alpha-amylase, β -glucosidase, endoxylanase, pectinase, or arabinofuranosidase activity was observed with AC1 (not shown).

3.3. Feeding trial 1: effect of AC1 on digestibility

No interactions were observed between DDGS and enzyme inclusion levels for viscosity of the digesta or AID of nutrients when pigs were fed corn-soy diets containing two different levels of DDGS and three different levels of AC1 (Table 2; $P > 0.10$). There was also no effect on viscosity associated with increasing DDGS inclusion from 15 % to 30 % (Table 2). However, supplemental AC1 had a tendency to decrease ($P = 0.092$) viscosity of distal jejunal digesta as a function of enzyme inclusion (Table 4). Increasing DDGS in the diet increased AID of crude fat ($P = 0.002$) and tended to decrease AID of NDF ($P = 0.075$) and ADF ($P = 0.054$). Supplemental AC1 tended to increase ($P = 0.076$) AID of NDF. There was no significant difference in feed intake across the treatments

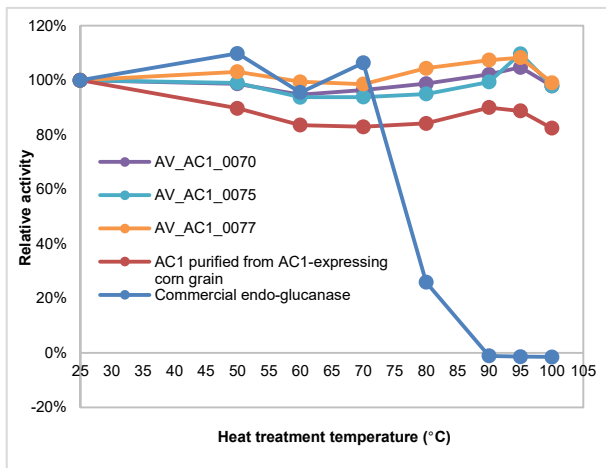


Figure 1. Thermostability of AC1. Relative glucanase activities after 5 min of incubation at different temperatures of three representative AC1 product batches (AV_AC1_0070, AV_AC1_0075, and AV_AC1_0077), AC1 that has been purified from AC1-expressing grain, and a commercial thermostable β-glucanase.

(data not shown), so enzyme consumption was a function of the inclusion rate in the diet.

3.4. Feeding trial 2: high-level dosing of AC1 in a corn-soy diet

In the enzyme treatment group, animals ingested on average 930 U/d AC1 from day 0 and day 21, and 2796 U/d from day 21 to day 42 (1866 U/d overall). There were no differences in body weight (BW) and average

daily gain (ADG) between pigs fed diets containing 2126 U/kg AC1 or the control diet without enzyme (Table 5). Similarly, there were no significant differences in average daily feed intake (ADFI) or gain-to-feed ratios (G:F) during any interval. These results indicate that inclusion of very high levels of AC1 does not adversely affect the performance of the animals.

Necropsies conducted at the end of the study revealed gross lesions in seven out of the forty pigs (two pigs from the group that were fed the diet containing 2126 U/kg AC1 and five pigs from the group that were fed the control diet). Briefly, the two pigs from the group that were fed AC1 presented thickened gastric mucosa and the presence of chyle in the mesenteric lymphovascular channels. Of the five pigs from the group that were fed the control diet, two also had thickened gastric mucosa; one had remnants of an umbilical hernia that had resolved on its own along with complications arising from the umbilical hernia; one had a number of lesions accompanied with a general failure to thrive, which were attributed to iron deficiency anemia; and one had suppurative omphalitis, with no evidence of entrance into the peritoneal cavity.

Blood samples from all animals were analyzed for both hematological characters (Table 6) and serum chemistry markers (Table 7). Results from these examinations showed no statistically significant differences between animals consuming the control diet or the diet containing 2126 U/kg of AC1 after 42 days.

4. Discussion

High-fiber containing ingredients such as DDGS are often included in swine diets as low-cost sources of protein, energy and phosphorus, and swine diets may include as much as 30–50 % DDGS, depending on the growth phase of the animal (U.S. Grains Council 2012). However, while DDGS has a greater gross energy than corn, swine can only utilize

Table 3. Enzymatic activities of protein extracts of grain derived AC1.

Enzyme activity (U/mg protein)	AC1, 37 °C		PC*, 37 °C		AC1, 80 °C		PC, 80 °C	
	Mean	SD**	Mean	SD	Mean	SD	Mean	SD
β-1,4-glucanase	12.120	12.060	0.140	0.004	281.520	10.925	1.189	0.056
Endo-cellulase	10.080	0.990	0.222	0.007	178.200	2.718	0.283	0.024
Cellobiohydrolase (Exocellulase)	0.192	0.004	0.001	0.000	0.907	0.015	0.000	0.000
Endo-β-1,3-glucanase	0.005	0.000	83.873	5.799	0.001	0.000	21.272	4.879
β-xylosidase	0.001	0.000	0.223	0.022	0.001	0.000	0.038	0.005

* PC, Positive controls: β-1,4-glucanase (Sigma G8673); Endo-cellulase (Sigma 22178); Cellobiohydrolase [Exo-cellulase, CBH I] (Megazyme E-CBH); Endo-β-1,3-glucanase (Megazyme E-LAMSE); β-xylosidase (Sigma X-3501).

** SD, standard deviation.

Table 4. Viscosity of distal jejunal digesta (cP¹) and apparent ileal of digestibility (AID) of DM, GE, crude fat, CP, NDF, and ADF in pigs supplemented with AC1 (based on units of β-1,4-glucanase activity) and distillers dried grains and solubles (DDGS), along with P-values to assess the effects of the DDGS inclusion, the enzyme inclusion (Enz) or the interaction of the two (DDGS × Enz).

DDGS ² , %	15			30			SEM	P value		
	0	150	450	0	150	450		DDGS	Enz	DDGS×Enz
Glucanase, U/kg										
Viscosity, cP	2.64	2.53	2.43	2.57	2.49	2.02	0.17	0.233	0.092	0.527
AID, %										
DM	73.67	74.36	75.45	73.21	74.14	75.16	1.13	0.730	0.266	0.994
GE	78.48	77.70	79.08	79.79	77.68	78.67	0.97	0.710	0.288	0.651
Crude fat	91.37	91.71	92.70	93.86	93.71	93.78	0.58	0.002	0.490	0.442
CP	84.27	83.33	85.14	85.39	84.86	85.63	0.93	0.176	0.387	0.854
NDF	44.17	52.43	52.61	43.30	46.47	46.66	2.87	0.075	0.076	0.597
ADF	42.87	33.38	39.94	32.98	30.91	35.43	3.46	0.054	0.181	0.546

¹ cP = centipoise (1 cP = 1/100 dyne s/cm²).

² DDGS: distiller's dried grains with solubles.

Table 5. Performance of pigs on untreated (control) and treated (AC1) diets.

	Control		AC1		P-value
	Mean	SD	Mean	SD	
BW (kg)					
d0	6.714	0.666	6.773	0.666	0.798
d21	10.877	2.203	11.250	1.658	0.549
d42	25.677	5.287	26.695	3.727	0.486
ADG (kg/d)					
d0-21	0.198	0.096	0.213	0.064	0.564
d21-42	0.704	0.156	0.736	0.118	0.474
d0-42	0.451	0.121	0.474	0.085	0.487
ADFI (kg/d)					
d0-21	0.809	0.174	0.795	0.138	0.848
d21-42	2.223	0.276	2.391	0.201	0.137
d0-42	1.509	0.209	1.595	0.135	0.286
G:F (kg/kg)					
d0-21	0.488	0.098	0.536	0.066	0.214
d21-42	0.638	0.038	0.614	0.042	0.196
d0-42	0.598	0.049	0.594	0.032	0.832

Table 6. Hematological characters in pigs after 42 days on the untreated (control) diet or the treated (AC1) diet.

Hematology	unit	normal range*		Control		AC1		P-Value
		Low	High	Mean	SD	Mean	SD	
WBC	$\times 10^3/\text{ul}$	11.0	22.0	15.407	3.089	15.783	3.020	0.707
RBC	$\times 10^6/\text{ul}$	4.87	8.19	6.570	0.460	6.605	0.514	0.822
Hemoglobin	gm/dl	8.1	14.7	11.311	1.285	11.779	0.518	0.149
Hematocrit	%	28.2	42.6	38.753	3.808	39.821	2.130	0.293
MCV	fl	43.4	64.5	59.195	5.985	60.495	3.900	0.433
MCH	pg	12.4	20.6	17.290	2.084	17.911	1.234	0.271
MCHC	gm/dl	27.4	35.8	29.158	1.243	29.595	0.721	0.194
RDW	%	14.9	32.4	22.116	3.612	20.505	1.831	0.092
Platelet	$\times 10^3/\text{ul}$	119	1000	415.950	117.605	449.210	98.733	0.351
MPV	fl	6.5	11.9	9.542	1.590	9.479	1.043	0.886
Neutrophil	$\times 10^3/\text{ul}$	2.0	11.9	4.400	1.383	4.961	1.635	0.261
Lymphocyte	$\times 10^3/\text{ul}$	4.0	17.9	9.653	2.757	9.562	1.871	0.906
Monocyte	$\times 10^3/\text{ul}$	0.0	3.7	0.752	0.365	0.726	0.200	0.788
Eosinophil	$\times 10^3/\text{ul}$	0.0	1.3	0.320	0.167	0.301	0.118	0.689
Basophils	$\times 10^3/\text{ul}$	0.0	1.6	0.128	0.051	0.122	0.044	0.660
Absolute LUC	$\times 10^3/\text{ul}$			0.153	0.092	0.111	0.080	0.137

* Normal range corresponds to reference intervals established by the Iowa State University Clinical Pathology Laboratory.

moderate amounts of dietary fiber for energy. Insoluble non-starch polysaccharides may have antinutritive effects, decreasing nutrient and energy availability, and reducing animal performance and carcass yields (Petry and Patience 2020). Therefore, there is considerable interest in using carbohydrate-degrading enzymes (carbohydrases) to improve the energy value of DDGS for swine. Two of the most commonly used carbohydrases in swine diets are xylanases and β -glucanases (Kiarie et al., 2017), although research on the use of these enzymes has shown inconsistent effects on animal performance (Bedford and Schulze 1998). While numerous studies have found benefits from formulating xylanases into high-fiber diets (Adeola and Cowieson 2014), some studies that compared glucanases and xylanases head-to-head have found that in some circumstances beta-glucanases better enhance the growth rate of weaned pigs on such diets (Bedford et al., 1992; Li et al., 2018).

AC1 is a thermostable β -glucanase with substantial cellulase and cellobiohydrolase activity. The thermostability of this enzyme is beneficial as the pelleting process during feed manufacture exposes feed ingredients to temperatures of 80 °C or higher for anywhere from 15 s to a few minutes. Enzymes produced by microbial fermentation frequently

include additional enzymes produced by the host organism. These are often poorly characterized and are often not thermostable (Adeola and Cowieson 2014). Since the “side activities” of AC1 were detected with purified enzyme, they are inherent to this one thermostable protein. Interestingly, the AC1 activity in crude grain extracts displays slightly greater thermostability than purified AC1. This observation suggests that factors present in the crude extracts (e.g. proteins, polysaccharides, small molecules) might augment the inherent thermostability of AC1, and removal of these factors during purification eliminates this effect.

The presence of fiber in feed can alter the viscosity of digesta in the gastrointestinal tract, thereby inhibiting the absorption of nutrients (Dikeman and Fahey 2006). Therefore, a key measure for the functionality of a carbohydrase in swine diets is to examine its effect on viscosity. In the current study, AC1 tended to decrease the viscosity of the digesta as a function of enzyme inclusion. In the absence of enzyme, the diet containing 30 % DDGS did not have higher viscosity than the diet containing 15 % DDGS, suggesting that the additional DDGS did not increase the amount beta-glucan enough to affect viscosity. In the current study, average viscosity of the digesta ranged from 2.02 cP to 2.64 cP. This

Table 7. Serum chemistry markers in pigs after 42 days on the untreated (control) diet or the treated (AC1) diet.

Chemistry	unit	normal range*		Control		AC1		P-Value
		Low	High	Mean	SD	Mean	SD	
ALT	IU/L	25.0	90.0	34.79	6.61	37.20	6.64	0.263
Sodium	mEq/L	135.0	150.0	139.53	1.98	139.65	1.63	0.832
Potassium	mEq/L	4.0	7.0	5.93	0.47	5.77	0.35	0.220
Chloride	mEq/L	95.0	110.0	100.53	1.39	101.05	1.73	0.306
Bicarbonate	mEq/L			31.37	2.03	30.45	1.79	0.142
Calcium	mg/dl	8.0	12.0	10.98	0.41	10.79	0.48	0.185
Phosphorus	mg/dl	4.5	9.0	9.94	0.70	10.30	0.89	0.177
Magnesium	mg/dl	1.82	3.65	2.36	0.28	2.40	0.16	0.631
BUN	mg/dl	6	30	9.11	2.40	9.45	1.79	0.613
Creat	mg/dl	0.5	2.7	0.90	0.12	0.86	0.11	0.248
Glucose	mg/dl	65.0	150.0	109.79	7.54	111.45	6.25	0.458
Total Protein	gm/dl	7.0	8.9	4.78	0.24	4.70	0.29	0.326
Albumin	gm/dl	3.0	4.5	3.42	0.34	3.33	0.33	0.379
AST	IU/L	10.0	100.0	46.00	23.90	55.70	45.77	0.416
Creatine Kinase	IU/L	100.0	2500.0	2368.42	2702.54	2327.68	2930.74	0.965
Alk Phos	IU/L	25.0	130.0	231.89	45.22	245.45	101.01	0.595
GGT	IU/L	10	100	52.42	15.31	49.20	13.84	0.495
Total Bilirubin	mg/dl	0.0	1.0	**		**		
Anion Gap				13.42	2.09	13.95	2.39	0.468
Lipemic Indice				20	0	20	0	
Hemolytic Indice				26.68	19.36	21.80	14.30	0.374
Icteric Indice				2	0	2	0	

* Normal range corresponds to reference intervals established by the Iowa State University Clinical Pathology Laboratory.

** Total bilirubin was below the LOD (0.10 mg/dl) in all samples at d42.

viscosity may have been too low to reveal any meaningful effect from enzyme addition, since previous researchers have suggested that it is difficult to identify an effect from exogenous enzymes when the viscosity of the digesta is below 10 cP (Bedford and Schulze 1998). This raises the question of whether diets incorporating an alternative source of dietary fiber such as barley or rye might demonstrate greater sensitivity to the effects of a glucanase. A study (Ayres et al., 2018) examining the effects of AC1 in broiler diets containing 10 % DDGS and found that AC1 decreased viscosity of the digesta. Poultry appear to be more sensitive to supplemental carbohydrases than swine (Adeola and Cowieson 2014), which may in part be due to the typically higher water content of the digesta in swine than in poultry, which makes swine less sensitive to changes in viscosity.

AC1 tended to increase the AID of NDF, while showing no effect on DM, GE, crude fat, CP or ADF. Others have seen varying effects on digestibility when carbohydrases were included in swine diets containing DDGS. For example, (Tsai et al., 2017) fed pigs corn-soybean meal diets supplemented with 30 % corn DDGS and found that a β -glucanase improved the apparent total tract digestibility (ATTD) of both NDF and ADF, but only during Phase 2 growth (d7-d21). Although the β -glucanase appeared to improve average daily gain during the period from day 21–35 relative to the negative control in this study, the enzyme had no effect on body weight, average daily feed intake or feed efficiency. Using pigs fed diets that included corn, soybean meal, corn DDGS and wheat middlings (Li et al., 2018), found that a blend of β glucanase and xylanase increased the ATTD of ADF but not NDF, DM, GE or CP. In this 28 day study, the enzyme blend improved average daily gain during the period from day 15–21 but had no effect on average daily feed intake or feed efficiency during any period. A number of factors likely contribute to the varying response that have been seen from including carbohydrases (and specifically β -glucanases) in swine diets containing DDGS. Among these are compositional differences in corn DDGS from different sources or crop years, and differences in the specific biochemical activities of

different enzymes that are labelled “glucanases” (Bedford and Schulze 1998).

In general, when consumed as part of the diet, enzymes, *per se*, are not toxic, since their effect in biological systems is primarily defined by their substrate specificity and catalytic activity. Carbohydrases in particular are unlikely to act directly on critical components of animal cells (Pariza and Cook 2010). In addition to their effects on the viscosity of the digesta, it has also been proposed that carbohydrases may benefit the animals because of the prebiotic properties of short oligosaccharides that are released through enzyme activity and the attendant changes in microbiota or immune responses of the pigs (Foo et al., 2017; Kiarie et al., 2017; Petry and Patience 2020). Given the range of possible effects that carbohydrases may have on animal health it is reasonable to assess how well animals tolerate a new enzyme as a feed additive (Schliffka et al., 2019). To test this, weaned pigs were fed either a typical corn/soy DDGS diet or a similar diet supplemented with 2126 U/kg AC1, which is 5–15 times the dose that was used in the first study described above, with the expectation that such a high dose would reveal any adverse effects of the enzyme. Effects on performance (body weight, average daily gain, average daily feed intake, and gain-to-feed ratio), as well as hematology characters and blood chemistry markers, which are sensitive to nutritional malabsorption, disease, and other physiological disorders (Clark and Coffey 2008; Schliffka et al., 2019) were all measured, and no significant differences were observed, indicating that the physiology of these animals was not altered by consuming the enzyme. In addition, all animals were monitored during the live phase for gross health effects as well as via necropsy at the end of the trial. While a small number of pigs displayed gross lesions during the necropsies, more of the affected animals were in the control group than in the enzyme-treated group, and none of the findings could be attributed to consumption of the enzyme. These results confirm that AC1 is well tolerated in the diets of young pigs and complement the results that demonstrated the tolerance of this enzyme in broiler diets (Broomhead et al., 2019).

Declarations

Author contribution statement

Philip A. Lessard: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Xuemei Li: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Jonathan N. Broomhead: Conceived and designed the experiments; Analyzed and interpreted the data.

Matthew H. Parker: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Christopher Bailey: Analyzed and interpreted the data.

R. Michael Raab: Conceived and designed the experiments.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare the following conflict of interests: Xuemei Li; [is currently employed by Takeda Pharmaceuticals]. All authors; [Are current or former employees of Agrivida, Inc.]. Xuemei Li and R. Michael Raab; [have patent applications pending related to the enzyme AC1].

Additional Information

No additional information is available for this paper.

Acknowledgements

The authors would like to thank Hongyu Chen and Dr. Sung Woo Kim (North Carolina State University), Melissa Malakowsky (RTI, LLC), Dale Waldner (Millbrook Feeds) and Dr. Jimmie Corley for contributions to the feeding trials.

References

Adeola, O., Cowieson, A.J., 2014. Board-invited review: opportunities and challenges in using exogenous enzymes to improve ruminant production. *J. Anim. Sci.* 92 (2), 427–442.

Ayres, V., Baldwin, H.L., Li, X., Xu, H., Raab, R.M., Boney, J.W., Broomhead, J.N., Moritz, J.S., 2018. The effect of corn-expressed carbohydrase on performance and

digesta viscosity of broilers fed a high non-starch polysaccharide diet. *J. Appl. Poult. Res.* 27 (4), 499–506.

Bedford, M.R., Schulze, H., 1998. Exogenous enzymes for pigs and poultry. *Nutr. Res. Rev.* 11 (1), 91–114.

Bedford, M.R., Patience, J.F., Classen, H.L., Inbarr, J., 1992. Effect of dietary enzyme supplementation of rye- and barley-based diets on digestion and subsequent performance in weanling pigs. *Can. J. Anim. Sci.* 72, 97–105.

Broomhead, J.N., Li, X., Raab, R.M., 2019. Safety of corn-expressed carbohydrase when fed to broilers at a high dietary level. *J. Appl. Poult. Res.* 28 (3), 631–637.

Clark, Sherrie G., Coffey, Natalie, 2008. Normal hematology and hematologic disorders in potbellied pigs. *Vet. Clin. Exot. Anim. Pract.* 11 (3), 569–582.

Cooper, Caitlin A., Moraes, Luis E., Murray, James D., Owens, Sean D., 2014. Hematologic and biochemical reference intervals for specific pathogen free 6-week-old Hampshire-Yorkshire crossbred pigs. *J. Animal Sci. Biotechnol.* 5 (1), 1–5.

Dikeman, Cheryl L., Fahey, George C., 2006. Viscosity as related to dietary fiber: a review. *Crit. Rev. Food Sci. Nutr.* 46 (8), 649–663.

Foo, Loon, Jee, Ling, Hua, Lee, Yung Seng, Chang, Matthew Wook, 2017. Microbiome engineering: current applications and its future. *Biotechnol. J.* 12 (3), 1–11.

Grains Council, U.S., 2012. Distiller's Dried Grains with Solubles (DDGS), 3rd. U.S. Grains Council, p. 406.

Jasek, A., Li, X., Xu, H., Raab, R.M., Broomhead, J.N., Lee, J.T., 2018. Effects of increasing concentrations of maize-expressed non-starch carbohydrase enzyme on broiler growth performance and ileal nutrient digestibility. *Int. J. Poult. Sci.* 17 (11), 543–551.

Kiarie, Elijah, Romero, Luis F., Nyachoti, Charles M., 2017. The role of added feed enzymes in promoting gut health in swine and poultry. *Nutr. Res. Rev.* 26 (2013), 71–88.

Li, Qingyun, Gabler, Nicholas K., Loving, Crystal L., Gould, Stacie A., Patience, John F., 2018. A dietary carbohydrase blend improved intestinal barrier function and growth rate in weaned pigs fed higher fiber diets. *J. Anim. Sci.* 96 (12), 5233–5243.

Myers, W.D., Ludden, P.A., Nayigihugu, V., Hess, B.W., 2004. Technical note: a procedure for the preparation and quantitative analysis of samples for titanium dioxide. *J. Anim. Sci.* 82 (1), 179–183.

NRC, 2012. Nutrient Requirements of Swine, eleventh ed. National Academies Press, Washington D.C.

Onasanya, Gbolabo O., Florence, O., Timothy, Oke, Sanni, M., Muhammad, Aliyu I., 2015. Parameters influencing haematological, serum and bio-chemical references in livestock animals under different management systems. *Open J. Vet. Med.* 5 (8), 181–189.

Pariza, Michael W., Cook, Mark, 2010. Determining the safety of enzymes used in animal feed. *Regul. Toxicol. Pharmacol.* 56 (3), 332–342.

Passos, Adami, Adson, Park, Inkyung, Ferket, Peter, von Heimendahl, Elke, Kim, Sung Woo, 2015. Effect of dietary supplementation of xylanase on apparent ileal digestibility of nutrients, viscosity of digesta, and intestinal morphology of growing pigs fed corn and soybean meal based diet. *Animal Nutr.* 1 (1), 19–23.

Petry, Amy L., Patience, John F., 2020. Xylanase supplementation in corn-based swine diets: a review with emphasis on potential mechanisms of action. *J. Anim. Sci.* 98 (11), 1–12.

Schliffka, Wolfgang, Heng Xiao Zhai, Estefania Pérez Calvo, Sabine van Cauwenberghe, Walsh, Maria C., Lopez-Ulibarri, Rual, 2019. Safety and efficacy evaluation of a novel dietary muramidase for swine. *Heliyon* 5 (10), 1–9.

Swiatkiewicz, S., Swiatkiewicz, M., Arczewska-Wlosek, A., Jozefiak, D., 2016. Efficacy of feed enzymes in pig and poultry diets containing distillers dried grains with solubles: a review. *J. Anim. Physiol. Anim. Nutr.* 100 (1), 15–26.

Tsai, T., Dove, C.R., Cline, P.M., Owusu-Asiedu, A., Walsh, M.C., Azain, M., 2017. The effect of adding xylanase or β -glucanase to diets with corn distillers dried grains with solubles (CDDGS) on growth performance and nutrient digestibility in nursery pigs. *Livest. Sci.* 197, 46–52.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74 (10), 3583–3597.