Early Food Safety Assessment of the Carbohydrase AC1 Protein

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1. Introduction

Cereal grains are broadly classified into two major categories, viscous and non-viscous cereals, depending on their content of soluble non-starch polysaccharides (NSP). Rye, barley, oats, and wheat contain considerable amounts of soluble NSP and are classified as viscous grains, whereas corn, sorghum, millet and rice contain negligible amounts of soluble NSP and are considered to be non-viscous cereals. Due to the high content of viscous NSP in grain of the former, feeds produced from these grains result in high viscosity of the digesta in the gastro-intestinal tracts of monogastric animals resulting in reduced digestibility of nutrients, negative impacts on the gut microbiome and other negative effects (Burnett, 1966; Choct and Annison, 1992; Bedford and Classen, 1992; Danicke et al., 1999). Since the 1980s glucanase and other enzymes that degrade soluble NSP have been added to the feed of monogastric animals to increase performance of animals fed diets based on grains with a high soluble NSP content (Hesselman and Åman, 1986; Campbell et al., 1989; Broz and Frigg, 1986; Newman and Newman, 1987). A large number of enzymes categorized as carbohydrases are approved for use in animal feed to depolymerize soluble NSPs and improve the digestion of nutrients in feeds based on grains high in soluble NSP content. These include glucanase as well as galactosidase, mannanase, pectinase, and xylanase. β-glucans are a primary soluble NSP of barley, wheat and other grains high in soluble NSPs and are present at levels of 3-4% in barley. β-glucan is a glucose polymer containing a mixture of β1-3 and β1-4 linkages that make its physicochemical properties different from cellulose that is a straight-chain glucose polymer with only β1-4 linkages. Four types of endo-acting glucanases , classified according to the type of glycosidic linkage they cleave, are capable of depolymerizing 1,3-1,4-β-D-glucan: endo-1,3-1,4-β -glucanases, endo-1,3(4)-β-glucanases, endo-1,4-β-glucanases and to a lesser extent endo-1,3-β-glucanases (McCarthy et al. 2003).

Although glucanases have been widely used in feeds based on grains high in soluble NSPs, their utility in corn-soybean meal based diets has also been demonstrated. NSPs in corn-soybean meal based diets have been shown to decrease the digestibility of nutrients by restricting access of digestive enzymes such as amylase and proteases to nutrients intertwined in fibrous cellular matrices (Cowieson, 2005). In addition, legume (e.g., soybean) NSPs are more complex in structure than those of cereals, containing a mixture of colloidal polysaccharides (galacturonans, galactan and arabinans). Accordingly, the addition of pectinase to a corn-soybean meal diet has been shown to significantly increase the metabolizable energy (ME) value of the diet. This improvement in the ME coincided with increased digestibility of galactose-rich polysaccharides (Kocher et al., 2002).

2. Name, Description, and Function of the Carbohydrase AC1

Agrivida, Inc. is developing animal feed enzyme products that are produced in the grain of maize. Genes encoding the enzymes under the regulation of monocot derived seed specific promoters are transformed into maize. The enzyme products produced in this manner will be marketed under the trade name of GraINzyme®. One of the GraINzyme® products under development by Agrivida, Inc. is a carbohydrase feed enzyme whose primary activity is endo-1,4-β-glucanase [redacted]. The gene encoding the GraINzyme® Carbohydrase AC1 enzyme was isolated from an environmental DNA library and encodes a 37 kDa protein. The genetic elements of the transformation plasmid [redacted] that contains the Carbohydrase AC1 gene that was used to transform maize are listed in Table 2 and depicted graphically in Figure 1. [redacted] the Carbohydrase AC1 protein consists of 317 amino acids [redacted]

Maize plants engineered to express this gene using Agrivida's GraINzyme® technology produce two to four hundred units of glucanase activity/gram of grain. The GraINzyme® Carbohydrase product referred to herein as Carbohydrase AC1 (AC1) will consist of coarse ground corn meal produced from maize plants expressing the glucanase gene in the grain. It will be included in relatively small amounts as a feed additive in poultry and swine diets in order to reduce the viscosity of digesta and improve the digestibility of major feed ingredients.

Table 1. Hydrolase activities in protein extracts of Carbohydrase AC1 producing grain are reported here. Enzyme assays were conducted at 37°C and [redacted] In each case the activity values shown are the average of three determinations and are expressed in arbitrary units. Control reactions incorporating commercially available enzymes with the specified activity were also run to demonstrate the functionality of each assay.

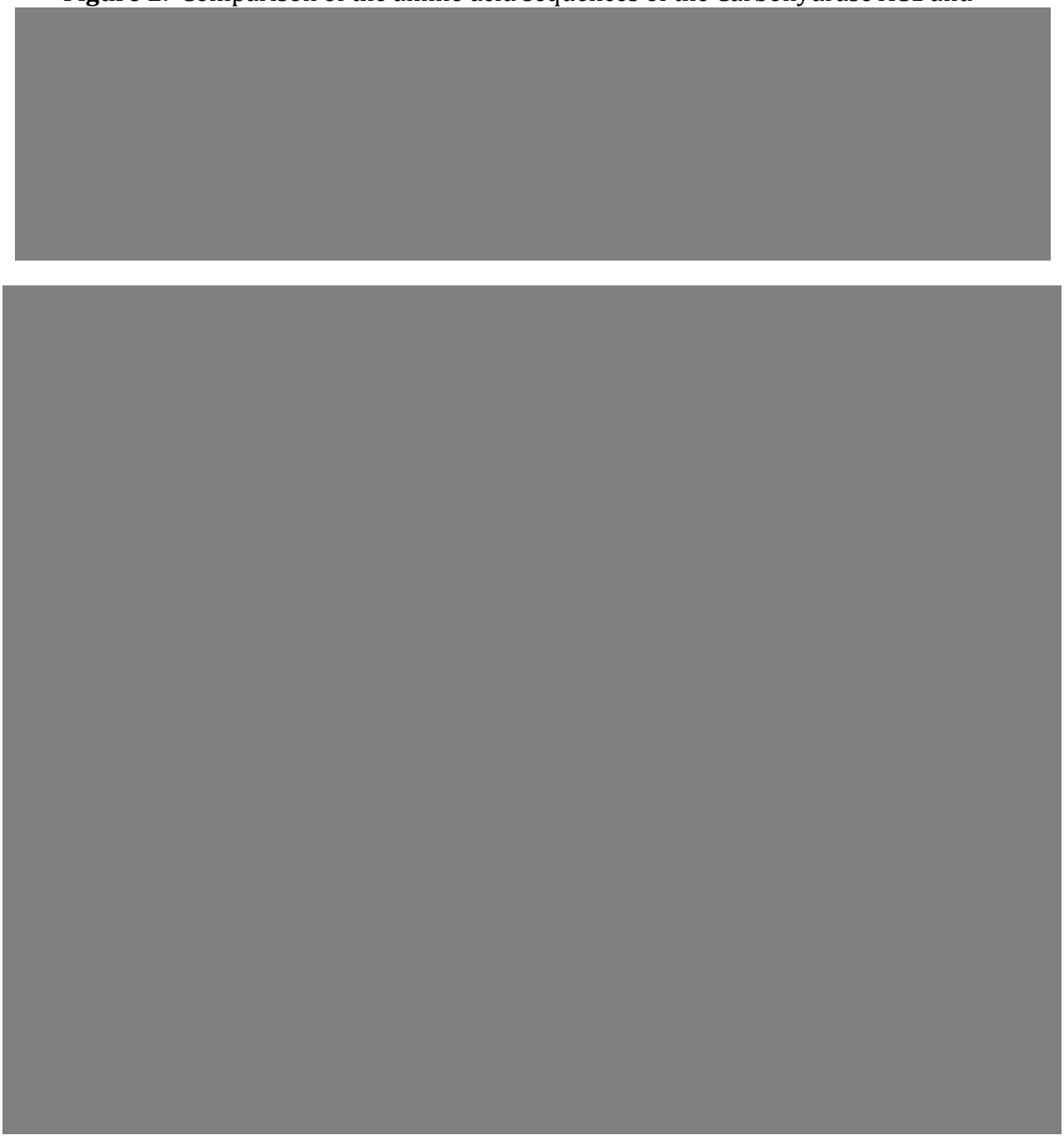
Enzymatic Activity	AC1, 37°C		Positive Control, 37°C		AC1, [redacted]		Positive Control, [redacted]	
	Mean	Std	Mean	Std	Mean	Std	Mean	Std
β-1,4-glucanase	0.031	0.003			0.267	0.013		
β-1,3-glucanase	0	0	1.177	0.051	0	0.002	0.219	0.002
[redacted]								

Figure 1. Genetic map of [redacted], the plant transformation vector containing the Carbohydrase AC1 gene that was used to create maize event [redacted] that produces the Carbohydrase AC1. The genetic elements shown on [redacted] are described in Table 2.

Table 2. Description of the genetic elements in plasmid [redacted]

Genetic Element	Description	Size (Kb)	Donor organism	Function	Reference
[redacted]					
PMI	Phosphomannose isomerase	1.1	*E. coli*	Plant selectable marker	Negrotto *et al.*, 2000
NosT	Nopaline synthase terminator	0.3	*Agrobacterium tumefaciens*	Transcriptional terminator/polyadenylation signal	Depicker *et al.*, 1982
LB	Left Border	<0.1	*Agrobacterium tumefaciens*	T-DNA boundary	Zambryski et al., 1982
aadA	aminoglycoside-adenyltransferase	0.8	*E. coli*	Bacterial selectable marker	Flung et al., 1985
sat	streptothricin acetyltransferase	0.5	*E. coli*	Bacterial selectable marker	Horinouchi et al., 1987
Psat	Streptothricin acetyltransferase promoter	0.2	*E. coli*	Transcriptional promoter for sat	Horinouchi et al., 1987
ori	Origin of replication in E. coli	<0.1	*E. coli*	Plasmid origin of replication	Itoh and Tomizawa, 1978
cos	cos site from bacteriophage lambda	<0.1	*E. coli* bacterio-phage lambda	Site of recombination into disarmed Ti plasmid	Collins and Hohn, 1978
RB	Right Border	<0.1	*Agrobacterium tumefaciens*	T-DNA boundary	Wang et al., 1984
[redacted]					
AC1	Carbohydrase AC1 coding sequence [redacted]	1	Environmental DNA library	Gene of interest; [redacted]	
[redacted]					
[redacted]					

Figure 2. Comparison of the amino acid sequences of the Carbohydrase AC1 and



3. Description of the Intended Effect of the AC1 Protein

The Carbohydrase AC1 is produced in the grain of maize but due to the relatively low content of water in grain it is not enzymatically active in the grain nor has it any obvious effect on the grain or the maize plant. The grain producing AC1 will be harvested and ground into a coarse meal that will be added as a feed additive at relatively low inclusion levels (500g to 10kg/ton of feed) to the feed of poultry and swine. The intended effect of the AC1 in animal feed is to improve the digestibility of feed in the animal's gastro-intestinal tract through the solubilization of NSPs in the diet thereby reducing the viscosity of the digesta and improving access of the animal's digestive enzymes to nutrients in the diet.

4. Identity and Source of Introduced Genetic Material

The gene encoding the GraINzyme® Carbohydrase AC1 enzyme was isolated from an environmental DNA library and encodes a 37 kilodalton (kDa) protein [redacted]

A transformation gene cassette containing the AC1 gene [redacted] Figure 1). The genetic elements of the T-DNA fragment that was used to transform maize and the individual genetic elements within the T-DNA fragment are described in Table 2 and presented in Figure 1. This plasmid was transformed by *Agrobacterium*-mediated transformation into immature corn embryo tissue as described by Negrotto *et al*. (2000) and transformants were selected based on the presence of the plant selectable marker *pmi* gene on the transformed DNA fragment that encodes the enzyme phosphomannose isomerase (PMI). The PMI enzyme enables corn tissue to grow on mannose as a sole source of carbon (Negrotto *et al*., 2000). The *pmi* gene has been used as a selectable gene in several genetically modified corn varieties that have completed review by the USDA, FDA, and EPA for food and feed safety, including corn events 5307 and Mir604 corn with resistance to corn rootworm, lepidoptera resistant Mir162, and α-amylase expressing 3272, all products of Syngenta Seeds. Corn plants containing the AC1 gene were cultivated and were found to produce two to four hundred units of carbohydrase activity per gram of grain.

5. Characterization of Maize Produced Carbohydrase AC1

The identity and characteristics of maize produced Carbohydrase AC1 have been confirmed by several different approaches. The AC1 protein was extracted from three representative Carbohydrase AC1 product batches that were produced by cultivation of AC1 expressing maize in three different locations (Table 3). Grain from each of the three product batches was ground to flour in a knife mill and protein extracts were prepared as follows: AC1 extraction buffer (100 mM sodium phosphate, 0.01% Tween 20, pH 6.5) [redacted] was mixed with 20 g of maize flour in a 250 mL flask. Duplicate samples from each product batch were prepared for extraction. Samples were shaken [redacted] for 1 hour. Approximately 1.2 mL of sample suspension was transferred into a 1.5 mL tube and centrifuged at 16,000 g for 10 minutes. The supernatant was used for the characterization of the AC1 protein.

Table 3. Planting locations and dates for the production of three representative Carbohydrase AC1 product batches and units of glucanase activity/g of grain flour.

Product Batch No.	AV AC1 0070	AV AC1 0075	AV AC1 0077
Planting Location	[redacted]		
Planting Date	11/28/2016	1/20/2016	11/28/2016
Harvest Date	4/27/2017	5/16/2016	4/27/2017
Units/g Flour	200.2	206.75	198.11

5.1. Glucanase activity in flour of AC1 expressing maize

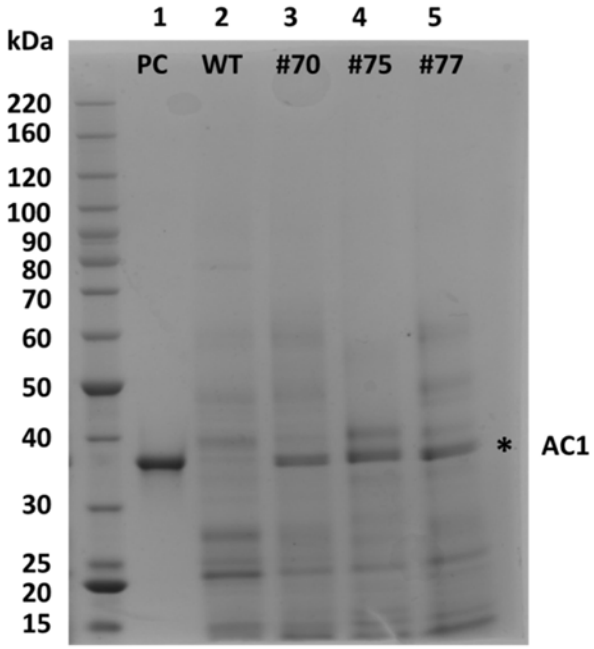
The Carbohydrase AC1 enzyme activity in each of the AC1 product batches was assayed according to a validated Agrivida, Inc. protocol that is described in Appendix 1. Since β-1,4 glucanase activity is the primary activity of the AC1 protein, a β-glucanase assay was used to determine the AC1 enzyme activity. The glucanase colorimetric assay described in detail in Appendix 1 uses a commercial substrate, azurine-cross linked barley β-glucan (AZCL-Beta-Glucan) from Megazyme (Wicklow, Ireland). Hydrolysis of this substrate by AC1 enzyme produces water soluble dyed fragments that can be measured by absorbance at 590 nm (A590) that is directly correlated to enzyme activity. The protein extracts produced from the AC1 grain flour samples as described above were diluted 60- and 80-fold and 50 µl of each dilution was mixed with 450 µl of AC1 extraction buffer containing the glucazyme substrate. The mixtures were incubated in a water bath [redacted] for 1 hour and mixed with 1 mL of 2% Tris base to terminate the reaction. The reaction was centrifuged at 3,000 g for 10 minutes, 100 µl of supernatant was removed to a microplate to measure absorbance at 590nm. AC1 enzyme activity (A590/mg) was determined for each batch by dividing the A590 by the amount of flour included in the protein extract (mg). The enzyme activity (A590/mg) can also be converted to

the activity units (unit/gram; Appendix 1). The amount of glucanase activity determined in the three AC1 product batches was about 200 units/g of maize flour (Table 3) compared to 0.08 units/g in wild-type maize flour.

5.2. Determination of the molecular weight of the AC1 protein

An SDS-PAGE gel containing protein extracts from each of the three test substances, protein extracts of corn flour derived from a conventional maize variety not engineered to produce AC1, and purified AC1 protein from AC1- expressing corn grain were stained with Coomassie blue to enable visualization of the proteins. Examination of the gel and comparison of the samples demonstrated that there is a prominent protein band in the extracts from all three test substances that is absent in the extract from the conventional corn flour and that has the same molecular weight as the AC1 protein that was produced and purified from AC1 producing corn grain (Figure 3). Comparison of the position of these protein bands in the gel relative to the protein molecular weight markers included on the gel shows that the prominent protein band in the extracts of the test substances and the purified AC1 protein are approximately 37 kilodaltons (kDa) in size. This estimation of the size of the protein bands compares well with the predicted size of 37.5~37.7 kDa (http://web.expasy.org/compute_pi) for the mature AC1 protein [redacted]

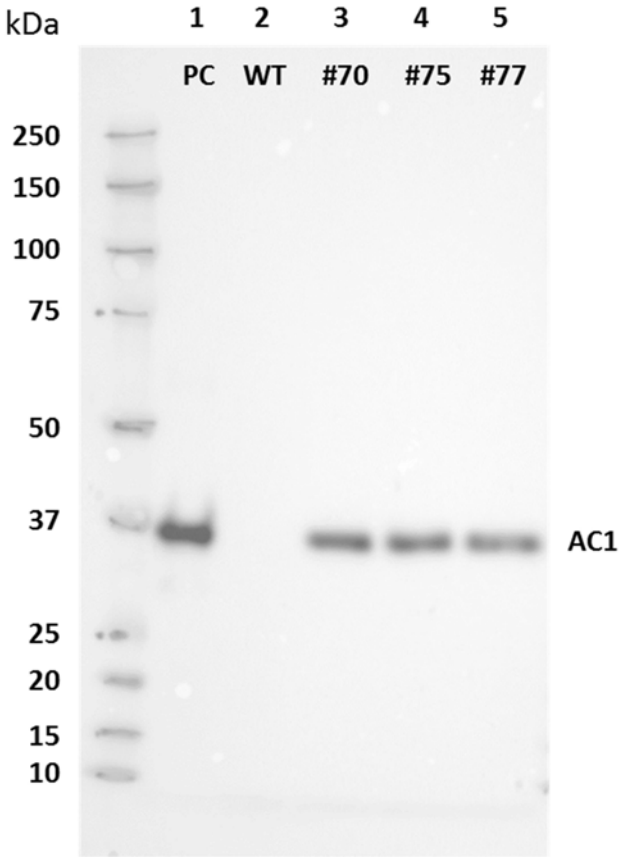
Figure 3. Coomassie-blue stained SDS-PAGE gel containing protein extracts from each of three AC1 product batches (AV_AC1_0070, #70; AV_AC1_0075, #75; and AV_AC1_0077, #77), extract from grain of a conventional non-AC1 expressing maize variety (WT, lane 2), and purified AC1 protein from AC1- expressing corn grain (PC, lane 1). Protein size markers (Invitrogen, Cat # 10747-012) were run in the left lane and their sizes in kDa are indicated on the left side of the gel. An asterisk indicates the position of AC1 protein present in aqueous extract from each of the AC1 product batches.



5.3. Immunoreactivity

Western blot analysis of the proteins in extracts from the three test substances was performed using mouse monoclonal antibody generated against the AC1 protein manufactured by [redacted] The results revealed the presence of one immunoreactive protein corresponding to the predicted molecular weight of the AC1 protein (*ca.* 37.7 kDa; Figure 4). Similarly, the antibody also reacted with the purified AC1 protein control. These results confirm the integrity and identity of the prominent protein species that are present in each of the three test substances but absent in conventional corn as AC1 and confirm its expected molecular weight of approximately 37 kDa.

Figure 4. Western blot of protein extracts that was reacted with an AC1- specific antibody. The samples on the Western blot include protein extracts of three independent AC1 production batches (AV_AC1_0070, #70; AV_AC1_0075, #75; and AV_AC1_0077, #77), the extract from grain of a conventional AC1 non-expressing maize variety (WT, lane 2) and purified AC1 protein from AC1 expressing corn grain (PC, lane 1). Protein size markers (Bio Rad, Cat # 161-0375) were run in the left lane and their sizes in kDa are indicated on the left side of the gel.



5.4. Confirmation of the Amino Acid Sequence of Maize Produced Carbohydrase AC1

As previously noted, the AC1 gene expression construct [redacted]

AC1 protein was purified from extracts of a representative AC1 product batch and the N-terminal amino acid sequence of the protein was determined by Edman degradation by Lake Pharma (Belmont, CA). These results confirm that the mature AC1 protein that is produced in the grain of maize has the N-terminal amino acid sequence that is expected from the coding sequence of the AC1 gene (Figure 2). Additional confirmation of the amino acid sequence of the grain-expressed AC1 protein was provided by tryptic digestion and LC/MS peptide mapping (also performed by Lake Pharma).

6. Assessment of the Allergenicity Potential of Carbohydrase AC1

6.1. Amino Acid Sequence Homology of AC1 to Known Protein Allergens

Bioinformatic analyses were conducted to evaluate the potential allergenicity of the Carbohydrase AC1 protein. The amino acid sequence of the mature AC1 protein was compared on 31 August 2017 to a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (version 17, January 18, 2017), which contains the amino acid sequences of known and putative allergenic proteins. Potential identities between the AC1 amino acid sequence and those of proteins in the allergen database were evaluated using the FASTA sequence alignment algorithm (Pearson and Lipman, 1988) with a scoring matrix = BLOSUM62, gap extension penalty = 2, and gap creation penalty = 12. The resulting alignments were returned and reviewed for identities greater than or equal to 35% over 80 or greater residues. None of the alignments met or exceeded the 35% threshold.

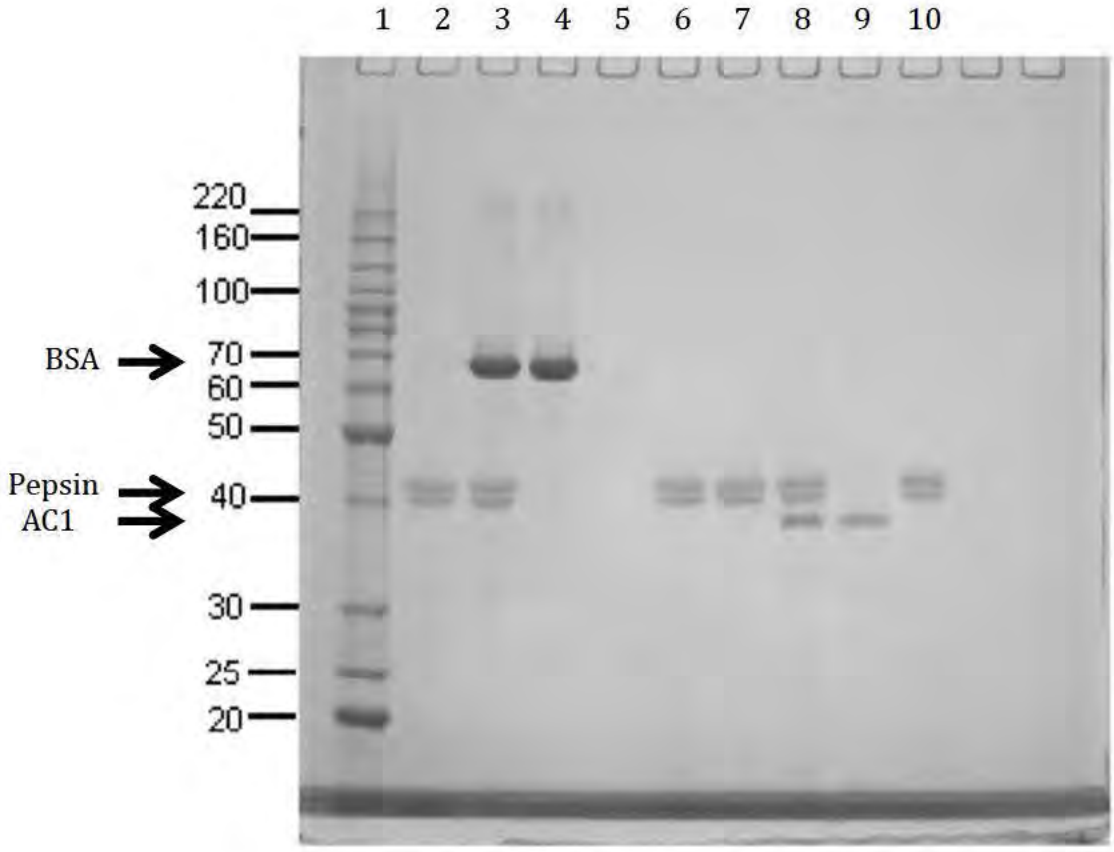
The Carbohydrase AC1 amino acid sequence was also evaluated for the presence of any eight or greater contiguous, identical amino acid matches to the same database of allergens noted above. The use of a match of eight contiguous, identical amino acids appears to have relevance based upon the minimum peptide length for an IgE-binding epitope (Metcalfe *et al*., 1996, Bannon and Ogawa, 2006). Results of the evaluation showed there were no contiguous identical amino acid matches of eight or more amino acids observed with the AC1 amino acid sequence. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the AC1 protein and known or putative protein allergens.

6.2. Lability of Carbohydrase AC1 to Pepsin in Simulated Gastric Fluid

One factor that is considered in relation to an assessment of potential allergenicity of novel proteins is their digestibility in a simulated gastric environment. Therefore, the susceptibility of purified, maize expressed, AC1 protein to proteolytic digestion by pepsin in simulated gastric fluid (SGF) was evaluated. The International Life Sciences Institute (ILSI) has standardized the pepsin digestibility assay protocol in a multi-laboratory evaluation (Thomas *et al*., 2004). The SGF formulation, and experimental parameters followed in the evaluation of the AC1 were similar to conditions used in the ILSI multi-laboratory evaluation. The reaction conditions and molar ratio of pepsin to AC1 protein in the study were similar to those described by Thomas *et al*. (2004).

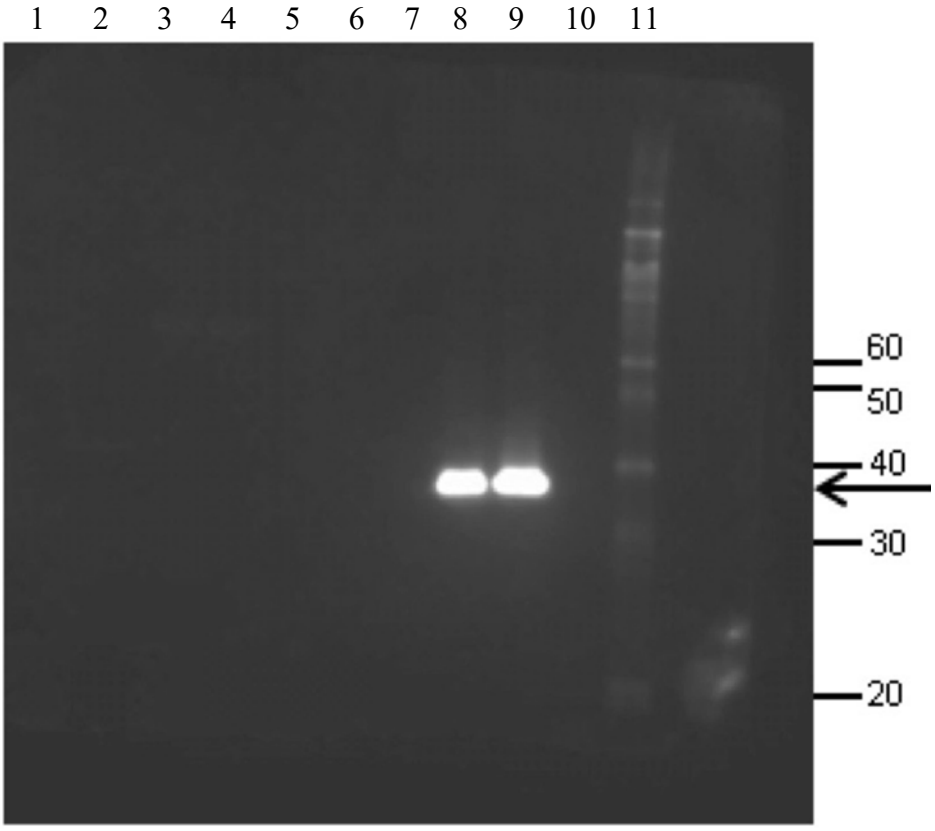
The Carbohydrase AC1 protein was incubated in SGF containing pepsin at pH 1.2 for 10 min. and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining of the proteins with Coomassie Blue. Results of the SGF study shown in Figure 5 demonstrate that the AC1 protein is not visible and is completely degraded after ten minutes in SGF containing pepsin at pH 1.2 (Figure 5, lanes 6 and 7). The AC1 protein is clearly visible in the samples with heat-inactivated pepsin or without pepsin (Figure 5, lanes 8 and 9, respectively). Aliquots of the reactions from the same SGF incubations were also subject to Western blot analysis (NuPage 4-12% Bis-Tris Gel). After electrophoresis the proteins were electro-blotted onto a membrane and a Western blot was performed using a mouse monoclonal AC1-specific antibody followed by a goat anti-mouse antibody. The results of the Western blot (Figure 6) confirm the previous results shown in the SDS-PAGE gel. After 10 min. of incubation in the SGF environment the AC1 protein was completely digested and could not be detected (Figure 6, lanes 6 and 7). These results demonstrate that the Carbohydrase AC1 protein is readily digested within 10 minutes in the SGF environment and therefore that it is unlikely to be a potential allergen.

Figure 5. Coomassie-Blue stained SDS-PAGE gel of the Carbohydrase AC1 protein after 10 minutes of digestion with pepsin in an SGF environment. The position of BSA and pepsin controls and the AC1 protein are indicated with arrows on the left. An explanation of the contents of the lanes is shown below.



Lane 1:	Protein molecular weight standards; sizes in kDa listed on the left
Lane 2:	BSA + Pepsin
Lane 3:	BSA + heat inactivated pepsin
Lane 4:	BSA
Lane 5:	Blank
Lane 6:	AC1 + pepsin
Lane 7:	AC1 + pepsin
Lane 8:	AC1 + heat inactivated pepsin
Lane 9:	AC1
Lane 10:	Pepsin

Figure 6. Western blot of the SGF reactions using a mouse monoclonal antibody specific to Carbohydrase AC1. The AC1 protein is clearly visible in lanes 8 and 9 (with heat inactivated pepsin and without pepsin, respectively) and is indicated by an arrow on the right. Lane 11 contains molecular weight standards of biotinlyated proteins whose sizes in kDa are indicated on the right.



Lane 1: Protein molecular weight standards
Lane 2: BSA + Pepsin
Lane 3: BSA + heat inactivated pepsin
Lane 4: BSA
Lane 5: Blank
Lane 6: AC1 + pepsin
Lane 7: AC1 + pepsin
Lane 8: AC1 + heat inactivated pepsin
Lane 9: AC1
Lane 10: Pepsin
Lane 11: Biotinylated protein molecular weight standards

6.3. Source of the Carbohydrase AC1 Gene and History of Exposure

As described in §4.0 the gene encoding the GraINzyme® Carbohydrase AC1 enzyme was isolated from an environmental DNA library and encodes a 37 kDa protein [redacted] (Figure 1) [redacted] owever, β-glucanase enzymes are ubiquitous in nature. In most plants they play a role in the opening of plasmodesmata channels that connect adjacent plant cells (Levy *et al*., 2007; Leubner-Metzger, 2003) and they are produced in the germinating seeds of plants (Vögeli-Lange *et al*., 1994), including tomato (Chun-Ta *et al*., 2001), barley (Leah *et al*., 1995), peanut (Liang *et al.* 2005), wheat (Moravčíková et al., 2016) and many others. In addition, β-1,3-glucanases attack critical components of fungal cell walls and are expressed in many plants as part of a broad generalized defense mechanism against fungal pathogenesis (Boller, 1987; Collinge and Slusarenko, 1987; Cornelissen and Melchers,1993). Therefore, it can be concluded that glucanase enzymes are present in many different plant derived food ingredients. β-glucanases from various sources are also widely used as food processing aids in the production of fermented beverages such as beer and wine and in the production of yeast extract.

As described in §1.0, β-glucanases and other carbohydrase enzymes have been used for many years in animal feed to increase the digestibility of the feed for monogastric animals (Bedford and Classen, 1992; Burnett, 1966). For the same purpose, glucanase enzymes are components of digestive aids marketed to humans and they appear in many such products, including Veganzyme® (Global Healing Center, 2017), Digestive Enzymes Ultra (Pureformulas, 2017), and βeta-1,3D Glucan (LifeExtension, 2017). As such, it can be concluded that there is a long history of safe consumption of glucanase and other carbohydrase enzymes in monogastric animals, including poultry, swine and humans.

6.4. Conclusions on the Allergenicity Potential of the Carbohydrase AC1 Protein

As described herein, all typical prognosticators of potential allergenicity of proteins indicate that the AC1 protein does not have the potential to be an allergenic protein. It has been demonstrated to be sensitive to digestion by pepsin in a simulated gastric environment and is completely digested within ten minutes in this environment. Bioinformatic analyses comparing its amino acid sequence to those of known allergens failed to demonstrate any close similarities that might indicate a potential for allergenicity. Clearly β-glucanase enzymes are not an allergenic class of proteins and are present in common human food and have been a part of the human diet for millennia and they have been consumed as dietary supplements for many years without any indications of allergenicity. Based on this information it is

concluded that the Carbohydrase AC1 has a very low allergenic potential.

7. Assessment of Toxicity Potential of the Carbohydrase AC1 Protein

7.1. Assessment of Amino Acid Homology of Carbohydrase AC1 to Known Protein Toxins

A global sequence similarity search of the AC1 amino acid sequence was conducted on May 25, 2017 against the NCBI Protein dataset using the BLASTP algorithm (Altschul et al., 2005). A sequence file comprising the translation of the AC1 gene was queried using the BLASTP 2.6.1 algorithm against the "nr" dataset, which incorporates non-redundant entries from all GenBank nucleotide translations along with protein sequences from SWISS-PROT, PIR, PRF, and PDB.

A cutoff expectation (*E*) score of 1.0 was used to generate biologically meaningful similarity between the AC1 protein and proteins in the "nr" dataset. Although a statistically significant sequence similarity generally requires a match with an *E* score of less than 0.01 (Pearson, 2000), a cutoff of $E < 1.0$ ensures that proteins with even limited similarity will not be overlooked in the search. Low complexity filtering was turned off and the maximum number of alignments returned was set to 5000.

The top 5000 proteins with homology to the Carbohydrase AC1 protein with an *E* score of less than 1.0 were examined. A large number of the accessions returned by the search displayed complete significance ($E = 0$) and represented nearly identical or closely related glucanase proteins from various microbial species. Most of the remaining sequences represented a variety of proteins that were all functionally related bacterial glycoside hydrolase enzymes such as endoglucanases and cellulases. None of the proteins were known toxins or proteins with toxicity related activities. This demonstrates that the AC1 protein is unlikely to share relevant sequence similarities with known protein toxins and is therefore unlikely to be toxic.

7.2. Evaluation of Toxicity of the Carbohydrase AC1

The Carbohydrase AC1 is a well-characterized enzyme with well-known hydrolase enzymatic activities. Very few proteins in nature are orally toxic and those that are toxic are so due to their specific biological mode of action. Enzymes in general and glucanase and related carbohydrase enzymes in particular are known not to be orally toxic. This is supported by the fact that humans have experienced oral exposure to glucanase enzymes as they are naturally present in most plants, including wheat, barley, peanuts, tomatoes and other food plants. Glucanase enzymes are also expressed in microbes such as yeasts and bacteria that are used in the production of common foods. In addition, glucanases are used in the processing of food products and they are marketed as human dietary supplements to improve digestion and there have been no reports or incidents of toxicity as a result of these exposures. As a result, it can be concluded that humans and animals have had a long

history of dietary exposure to glucanase enzymes without any indication of toxicity. The only biological activity of the Carbohydrase AC1 is its carbohydrate hydrolysis activity on NSP substrates, and the only other dietary impact of its presence in food is the same as any other dietary protein that is digested to its constituent amino acids. A search of the nonredundant protein databases with the AC1 amino acid sequence revealed that the AC1 protein has no similarity to any known toxic proteins.

With consideration of the above points, the decision tree for establishing the safety of food processing enzymes as described by Pariza and Johnson (2001) was applied to the Carbohydrase AC1. The results of this analysis are presented in Table 4. Since glucanases have a long history of safe use in animal feed and in human dietary supplements and since the maize production host is known to be safe, the decision tree determined that the Carbohydrase AC1 is safe and that animal toxicity studies are not required to demonstrate its safety as a food or feed enzyme. In conclusion, there is sufficient information about the Carbohydrase AC1 to conclude that it is safe for its intended use as a feed additive for poultry and swine. Furthermore, its presence in small amounts in human food would also not present any human safety or health concerns.

7.3. Conclusions on Toxicity Potential of Carbohydrase AC1

Bioinformatic comparison of the amino acid sequence of AC1 to the nonredundant protein database in NCBI using BLASTP algorithm demonstrated that the AC1 protein does not have significant homology to known toxic proteins. In addition, there is a long history of safe consumption of glucanase enzymes by humans and animals since glucanases are ubiquitous in plants and since they are consumed by humans and animals in the form of dietary supplements and feed additives, respectively. Application of the decision tree developed by Pariza and Johnson (2001) to determine the safety of food processing enzymes to the Carbohydrase AC1 demonstrated that the Carbohydrase AC1 is considered to be safe. Considering all of these factors, it is concluded that the Carbohydrase AC1 has very low potential to be a toxic protein.

Table 4. Application of the decision tree for determining the safety of food processing enzymes that was developed by Pariza and Johnson (2001) to the Carbohydrase AC1.

	Question?	AC1
1	Is the production strain genetically modified? If yes go to 2.	YES
2	Is the production strain modified using rDNA? If yes go to 3a.	YES
3a	Do the enzyme products have a history of safe use in food or feed? If yes go to 3c.	YES
3c	Is the test article free of transferable antibiotic resistance gene DNA? If yes go to 3e.	YES
3e	Is all other introduced DNA well characterized and free of attributes that would render it unsafe? If yes go to 4.	YES
4	Is the introduced DNA randomly integrated into the genome? If yes go to 5.	YES
5	Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotrophic effects which may result in the synthesis of toxins will not arise due to the genetic modification? If yes go to 6.	YES
6	Is the production strain derived from a safe lineage? If yes the test article is ACCEPTED	YES

8. Conclusions

According to the guidance of the FDA for the early food safety evaluation of new proteins in new plant varieties that are under development, the Carbohydrase AC1 protein was evaluated for its allergenicity and toxicity potential.

The allergenic potential of AC1 was assessed by: 1) bioinformatic comparison of the amino acid sequence of the AC1 protein with the amino acid sequences of well known protein allergens; 2) evaluation of the stability of the maize produced, purified AC1 protein in a simulated gastric environment; and 3) assessment of the AC1 gene source and history of use or exposure.

Bioinformatic analyses revealed no similarities between known protein allergens and the AC1 protein sequence. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the AC1 protein and known allergens. The Carbohydrase AC1 protein was completely degraded within 10 minutes in the simulated gastric fluid environment. Further, there has been a long history of human dietary exposure to similar glucanase enzymes. Taken together, this information supports the conclusion that the Carbohydrase AC1 protein is not a potential allergen.

Bioinformatic analyses revealed Carbohydrase AC1 to be similar to other microbial and plant derived carbohydrase proteins. No biologically relevant sequence similarities were detected between known protein toxins and the AC1 protein. These data support the conclusion that the AC1 protein is not toxic.

Based on the data and information provided in this document, Agrivida, Inc. has determined that the AC1 protein is unlikely to cause an allergic reaction in humans or be toxic upon consumption by either humans or animals.

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10. Appendices

10.1 Appendix 1: Carbohydrase AC1 Activity Colorimetric Assay Validation

Carbohydrase Colorimetric Assay

The procedure for measuring the activity of carbohydrase enzymes, including β-glucanase using an azo-barley-glucan as a substrate has been described by McCleary and Shameer (1987). This *in vitro* assay produces a blue-colored solution by hydrolyzing the substrate, changing the color of the reaction supernatant, which can be recorded as a change in absorbance at 590 nm. The rate of the color change is correlated directly to enzyme activity.

This *in vitro* colorimetric glucanase enzyme activity assay has proven to be a simple and reliable method. The enzyme activity data obtained from this method correlates well with the *in-vivo* response to enzyme supplementation of barley-based diets when fed to young chicks (Rotter et al., 1990).

The glucanase colorimetric assay described herein uses a similar commercial substrate, azurine-cross linked barley β-glucan (AZCL-Beta-Glucan) from Megazyme (Wicklow, Ireland). Hydrolysis of this substrate by Agrivida GraINzyme® Carbohydrase AC1 enzyme produces water soluble dyed fragments, and the rate of release of these dyed fragments, which increases in absorbance at 590 nm, correlates directly to enzyme activity (Megazyme, 2012).

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1. Limit of Detection (LOD)

The limit of detection for the AZCL-barely-beta glucan assay was determined by calculating the mean A590 and standard deviation of the mean for multiple negative samples, each of which was derived from corn that does not produce Carbohydrase AC1 ("WT corn"). The LOD for the assay can be defined as the mean A590 of these negative samples plus three standard deviations.

Table 1. Data from assays of WT corn samples and calculations for LOD (mean A590 plus 3 standard deviations)

Negative Seed A590 Readings											
0.144	0.097	0.115	0.094	0.091	0.093	0.099	0.097	0.093	0.087	0.086	0.088
0.095	0.101	0.111	0.087	0.095	0.086	0.091	0.086	0.088	0.084	0.085	0.089
0.085	0.084	0.085	0.09	0.083	0.085	0.097	0.087	0.089	0.09	0.08	0.077
0.087	0.099	0.084	0.087	0.087	0.087	0.105	0.103	0.094	0.092	0.084	0.078
0.078	0.085	0.081	0.116	0.097	0.09	0.108	0.092	0.094	0.088	0.083	0.104
0.086	0.09	0.083	0.087	0.096	0.095	0.09	0.086	0.098	0.084	0.076	0.094
0.099	0.163	0.125	0.092	0.08	0.079	0.084	0.077				

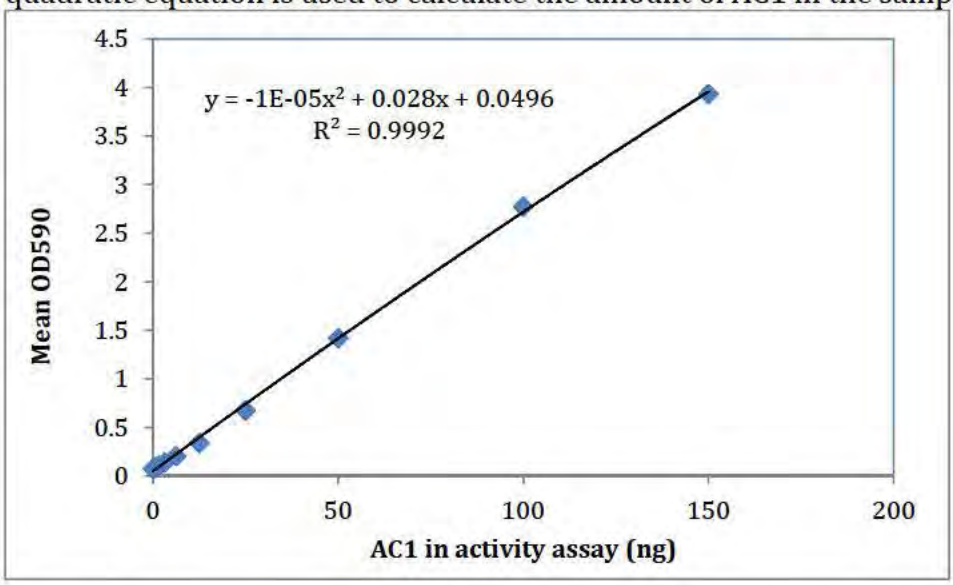
n =	Mean OD	Std dev	3 std dev	LOD
80	0.092263	0.013513	0.040539	0.132801

By comparing the A590 of the LOD to a standard curve of AC1 calibrators, it is possible to express the LOD in terms of nanograms of AC1 protein. A series of 12 dilutions of AC1 were used in replicate assays involving AZCL-barley-beta-glucan (as described in Appendix 2), and the A590 values from each sample were recorded (Table 2). Applying a quadratic best-fit curve to the data created a standard curve that can be used to infer the correlation between A590 and ng of AC1 protein (Figure 1).

Table 2. Data for calculating the standard curve.

Activity of the calibrators (A590)													
AC1 in activity assay (ng)	0	0.3	0.5	1.0	1.6	3.1	6.3	12.5	25.0	50.0	100.0	150.0	200.0
Rep 1	0.077	0.08	0.088	0.098	0.11	0.142	0.212	0.348	0.709	1.48	2.825	3.961	Plateaued
Rep 2	0.077	0.076	0.084	0.094	0.105	0.133	0.195	0.331	0.641	1.364	2.725	3.91	Plateaued
Average	-3E-08	0.078	0.086	0.096	0.108	0.138	0.204	0.340	0.675	1.422	2.775	3.936	

Figure 1. Standard curve of AC1 activity vs the amount of AC1 in the assay. The quadratic equation is used to calculate the amount of AC1 in the sample.



Using the quadratic equation for the best-fit standard curve, the LOD value of 0.132801 A590 can be converted to 2.97 ng of AC1 protein.
From these results, we infer that the LOD for the GraINzyme® Carbohydrase AC1 activity detection is about 3 ng.

2. Assay Sensitivity and Matrix Effects

To determine whether the beta-glucanase activity colorimetric assay is affected by the matrix when AC1-expressing corn product is mixed with animal feed, AC1 protein was assayed in the presence of either WT (non-AC1) corn flour, or the formulated animal feed.

3. Mixing and Recovery of AC1 activity from WT corn product

[redacted]

Table 3. Data from assay of AC1 in the presence of ground WT corn.

AC1 spiked in extraction buffer	AC1 in activity assay	Extraction Buffer (A590)			Product Matrix (A590)			% Average Recovery
ppb	ng	Replicate_1	Replicate_2	Replicate_3	Replicate_1	Replicate_2	Replicate_3	(Matrix/Buffer)
0	0	0	0.002	0	0	0	0	0.00%
2.5	0.25	0	0.003	0.001	0	0	0	0.00%
5	0.5	0.003	0.003	0.004	0.009	0.01	0.011	300.00%
12.5	1.25	0.02	0.023	0.017	0.026	0.036	0.021	138.33%
25	2.5	0.029	0.03	0.027	0.045	0.047	0.047	161.63%
35	3.5	0.064	0.067	0.06	0.078	0.082	0.073	122.15%
62.6	6.25	0.102	0.109	0.11	0.115	0.138	0.147	125.09%
125	12.5	0 26	0.269	0.267	0.309	0 284	0.29	110.95%
250	25	0.527	0.599	0.575	0.655	0.61	0.591	109.30%
500	50	1.276	1.341	1.311	1.406	1.428	1.391	107.58%
1000	100	2.525	2.724	2.499	2.793	2.666	2.454	102.27%
1500	150	3.888	Plateaued	Plateaued	Plateaued	Plateaued	3.682	94.70%

The recoveries ranged between 125% and 95% for samples with AC1 protein between 3.5 and 150ng. At 3.5ng, which was close to the LOD, the average recovery was about 122% suggested that very little product matrix effect was present. Samples with 1.25 ng enzyme or lower resulted in recoveries ranging between 138% and 300%, suggesting the assay is less sensitive at such low enzyme inclusion.

4. Spiking and Recovery of AC1 activity from feed

To determine whether a complex feed mixture, such as that used in many poultry diets, might interfere with the sensitivity of the AC1 assay, serial dilutions of the enzyme were again prepared in extraction buffer and mixed with samples of feed, then assayed for recoverable enzyme activity. For the feed sample, a control (basal) diet (no AC1 addition) from a poultry feeding trial, was milled to less than 1.0 mm particle size. For the serial dilutions, extraction buffer was spiked with AC1 protein at concentrations of 2.5, 5, 10, 25, 50, 70, 125, 250, 500, 1000, and 1500ppb, and 2.5ml of each buffer was mixed with 0.5g of feed sample in triplicate. The remaining spiked buffer and the buffer/feed mixes were placed on a temperature-controlled shaker, shaking for 1 hour at 250rpm and [redacted]. 100ul of supernatant ("extract") was removed from each sample and was used in the standard assay as described in Appendix I. The amount of AC1 protein used in enzyme assay was 0.25, 0.5, 1.25, 2.5, 3.5, 6.25, 12.5, 25, 50, 100 and 150ng, respectively. AC1 enzyme recovery is the average of triplicate assay activities from the feed matrix relative to that from AC1 spiked buffer.

Table 4. Data from assay of AC1 in the presence of poultry feed.

AC1 spiked in extraction buffer	AC1 in activity assay	Extraction Buffer (A590)			Feed Matrix (A590)			% Average Recovery
ppb	ng	Replicate_1	Replicate_2	Replicate_3	Replicate_1	Replicate_2	Replicate_3	(Matrix/Buffer)
0	0	0	0	0	0	0	0	0.00%
2.5	0.25	0.001	0.006	0.003	0	0	0	0.00%
5	0.5	0.007	0.01	0.007	0	0	0	0.00%
12.5	1.25	0.02	0.022	0.019	0.006	0	0	9.84%
25	2.5	0.051	0.053	0.05	0.05	0.053	0.051	100.00%
35	3.5	0.116	0.112	0.115	0.117	0.109	0.104	96.21%
62.6	6.26	0.278	0.284	0.264	0.26	0.28	0.268	97.82%
125	12.5	0.287	0.265	0.264	0.334	0.308	0.312	116.91%
250	25	0.521	0.504	0.496	0.552	0.568	0.58	111.77%
500	50	1.313	1.259	1.168	1.408	1.327	1.259	106.79%
1000	100	2.395	2.418	2.401	2.793	2.642	2.59	111.24%
1500	150	Plateaued	Plateaued	3.762	Plateaued	Plateaued	Plateaued	Plateaued

*Plateaued: these values were above the maximum detection threshold of the spectrophotometer.

From these observations, recoveries of AC1 from samples that included between 2.5 and 100ng enzyme ranged between 96% and 117% in the presence of ground feed. When the enzyme was included at 2.5 ng, the average recovery was 100% suggesting that presence of the feed matrix had very little effect on the sensitivity of the assay. Samples that contained 1.25ng of enzyme or less resulted in poor recovery, suggesting the assay is less sensitive at such low levels of AC1 inclusion. Based on the data from experiments in which AC1 activity was measured after mixing the enzyme with milled corn or feed, the LOQ of 3.5 ng is confirmed.

5. AC1 enzyme activity recovery from the formulated feed diets

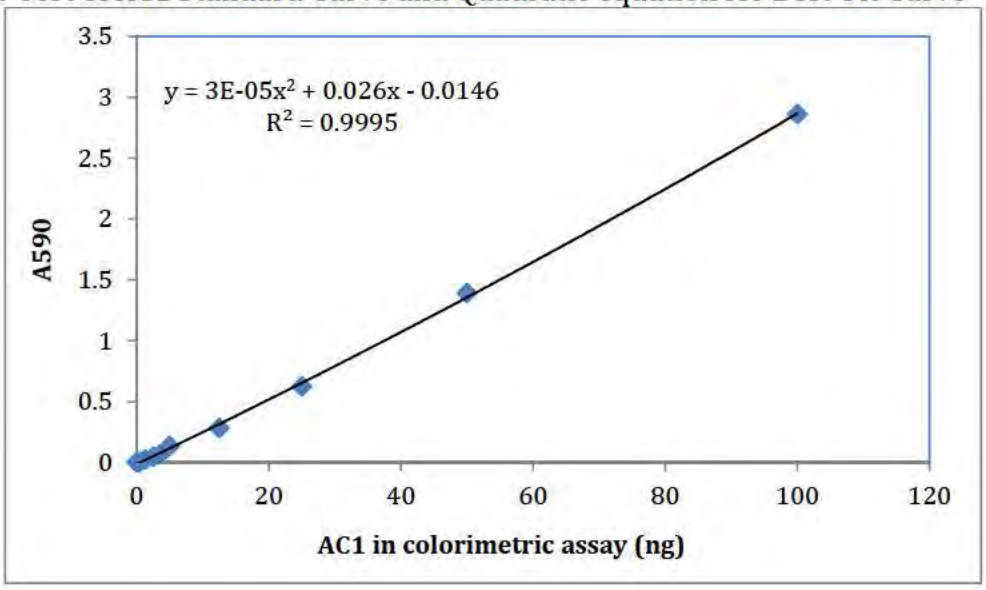
To test whether the glucanase colorimetric assay can reliably detect AC1 enzyme activity from industrial-type diets formulated with ground AC1-expressing corn meal, the AC1 activity in the diets was firstly compared with that from a standard curve of AC1 protein (Table 5 and Figure 2) to determine the amount of AC1 protein presented in each diet (Table 7). Then the AC1 content in the aqueous extracts from these diets was quantified with a proprietary ELISA plate (Table 6). The amount of AC1 protein detected by these two methods was compared to determine the reliability of the glucanase colorimetric assay method (Table 7).
To generate the standard curve, as stated in section 10.2.2, AC1 protein was mixed with extraction buffer at 2.5, 5, 12.5, 25, 35, 62.5, 125, 250, 500, 1000 and 1500ppb, and 2.5ml of each buffer was mixed with 0.5g of the control diet (no AC1 addition). The amount of AC1 protein used in each enzyme assay was 0.25, 0.5, 1.25, 2.5, 3.5, 6.25, 12.5, 25, 50, 100 or 150ng in 100ul of protein extract from control diet. The sample extraction and activity assay was carried out according to the standard protocol.

A standard curve of A590 values reflecting enzyme activity recoveries from the spiked feed vs. the nanograms of AC1 used in the enzyme activity assay is shown below (Table 5 and Figure 2).

Table 5. Data from AC1 Standard Curve

The amount of AC1 protein in the extracts from the spiked control diet was added to enzyme assay												
(ng)	0	0.25	0.5	1.25	2.5	3.5	5	12.5	25	50	100	150
A590	0	0	0.004	0.021	0.046	0.065	0.136	0.281	0.624	1.391	2.861	Plateaued

Figure 2. Plot of AC1 Standard Curve and Quadratic equation for Best-Fit Curve



A poultry feeding trial was conducted with an industry type starter diet formulated with 0, 5, 50, 100, 200 and 400 units corn-expressed AC1 per kilogram of diet (see below for the definition of units). After preparing the diets, each was ground by a knife mill, protein was extracted, and AC1 enzyme activity was assayed according the standard protocol in Appendix 2.

The amount of AC1 protein in the extract was quantified with a proprietary ELISA plate as listed in the Table 6 below. The ELISA assay protocol is presented in Appendix 3.

Table 6. AC1 Detection by ELISA

Formulated Diets	Target Dose Unit/kg	ELISA Detected AC1 in protein extract ng/mL	
		Ave	Std
Diet_1	0	1.89	0.34
Diet_2	5	2.61	0.78
Diet_3	50	45.14	7.23
Diet_4	100	103.88	15.66
Diet_5	250	220.50	41.26
Diet_6	500	475.90	158.53

AC1 activities measured via the colorimetric (A590) assay in extracts from the formulated diets of the feeding trial are shown in Table 7 below. For example, in Diet_3, which was formulated with a target dose of 50 unit AC1/kg, the activity of 0.1±0.006 A590 was detected. This A590 value is converted to 4.39ng based on the standard curve, and this number is very close to the AC1 protein that was detected by ELISA (4.51ng). In Diet_4, which was formulated with a target dose of 100 unit AC1/kg, an A590 value of 0.24±0.033 was obtained which can be converted to 9.68ng from the standard curve, and this number is very close to the AC1 protein detected by ELISA study (10.39ng).

Table 7. Detection of AC1 in the formulated feed

Formulated Diets	Target Dose Unit/kg	A590		ng of AC1 in assay	
		Ave	Std	Converted from the Std Curve	Based on ELISA Assay
Diet_1	0	0.002	0.002	0.64	0.19
Diet_2	5	0.002	0.000	0.64	0.26
Diet_3	50	0.100	0.006	4.39	4.51
Diet_4	100	0.240	0.033	9.68	10.39
Diet_5	250	0.644	0.071	24.63	22.05
Diet_6	500	1.308	0.037	48.19	47.59

From these results, it is concluded that AC1 activity can be detected reliably in formulated poultry diets via the colorimetric assay and that the results correlate well with those of an AC1-specific ELISA assay.

6. Converting colorimetric absorbance values to activity units

One Unit of Carbohydrase AC1 activity is defined as the amount of enzyme required to release one micromole of reducing sugar equivalents such as glucose from 1% barley-β-glucan, at pH6.5 and [redacted].

The β-glucanase unit (BG unit) activity assay involves multiple steps (as described in Appendix 4) and is less accurate with feed samples, especially when the target dose is lower than 300 unit/kg. This is probably due to the presence of endogenous enzyme activities and reducing sugars in feed, which can interfere with the unit

assay. As the colorimetric assay is relatively simple, sensitive and reliable, we use colorimetric absorbance values to infer unit values, and will market the Carbohydrase AC1 product using the BG unit as a descriptor of activity. To determine the mathematical relationship between values from the colorimetric and unit assays for AC1, we prepared a serial dilution of AC1 protein, and measured the activity via both assays. The amount of AC1 protein was also measured via ELISA so that both activities could be correlated as a function of amount of AC1 protein. AC1 protein dilutions were first measured in the AC1 colorimetric assay and then via ELISA (Table 8), and the amount of enzyme activity was calculated per mg AC1 protein. Averaging all 10 measurements showed AC1 activity was about 50,461 ± 11,347 A590/mg AC1.

Table 8. Enzyme activities of a serial dilution of AC1 protein from colorimetric assay and the protein concentrations in the diluents measured by ELISA.

ng of AC1 in colorimetric assay	AC1 Activity (A590)	mg of AC1 in the assay based on ELISA data	A590/mg AC1
0.5	0.011	3.06102E-07	35,935.77
1	0.024	6.74523E-07	35,580.68
2	0.049	9.69415E-07	50,545.93
3	0.066	1.42217E-06	46,407.94
4	0.1175	2.21927E-06	52,945.22
5	0.1385	3.15029E-06	43,964.17
10	0.3	6.72132E-06	44,634.09
20	0.668	1.0013E-05	66,713.31
25	0.9355	1.46043E-05	64,056.41
40	1.5875	2.4871E-05	63,829.31

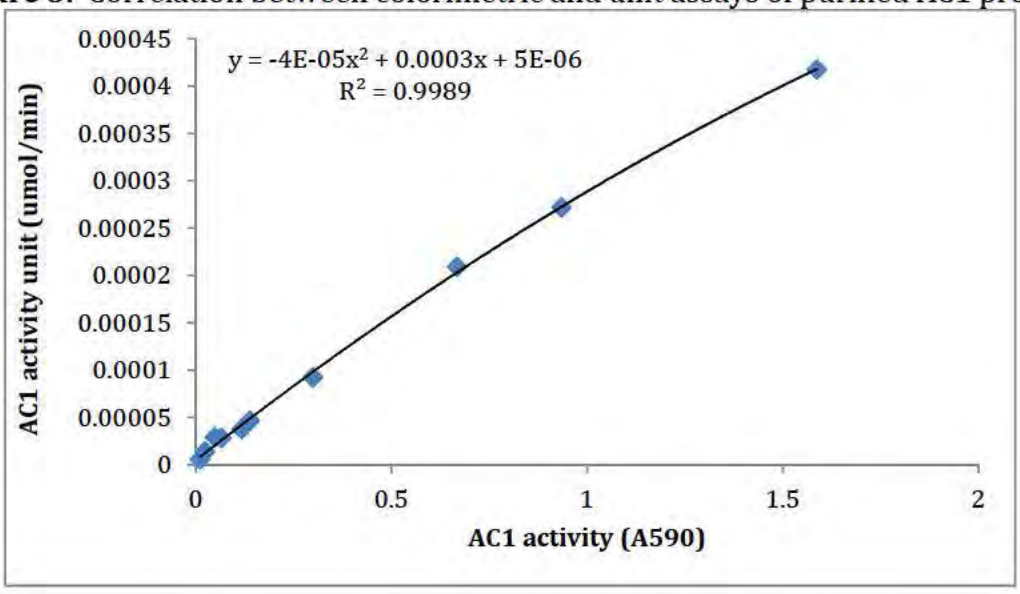
When these same dilutions of AC1 protein were used in the activity unit assay (Appendix 4), AC1 activity was calculated as the values of μmol of glucose released per minute per gram of AC1, and the average activity is about 456,461 ± 109,990BG unit/g AC1.

Table 9. Enzyme activities of a series diluted AC1 protein from BG unit assay and the protein concentrations in the diluents measured by ELISA.

ng of AC1 in colorimetric assay	Activity (umol/min)	g of AC1 in A590 assay based on ELISA	unit/g AC1
0.5	5.49891E-06	3.06102E-10	431,143.62
1	1.39237E-05	6.74523E-10	495,415.12
2	2.92756E-05	9.69415E-10	724,781.00
3	2.81523E-05	1.42217E-09	475,086.76
4	3.75132E-05	2.21927E-09	405,680.16
5	4.63124E-05	3.15029E-09	352,823.56
10	9.22432E-05	6.72132E-09	329,375.19
20	0.00020888	1.0013E-08	500,661.15
25	0.000271785	1.46043E-08	446,637.97
40	0.000417628	2.4871E-08	403,001.83

Comparing the results from the two AC1 assays, on average 1 A590/mg AC1 corresponds to about 9 units/g AC1.
Directly comparing the results from the colorimetric and unit assays of purified proteins shows that these two assays are well-correlated, as shown in Figure 3.

Figure 3. Correlation between colorimetric and unit assays of purified AC1 protein.



To test whether the relationship between the colorimetric and unit assays was preserved in more complex samples, both assays were used to measure AC1 activity in grains with different levels of AC1 protein expression. Table 10 illustrates glucanase activity data from AC1 corn products using both the colorimetric assay

and the unit assay. The average ratio between unit/g value and A590/mg value is about 9, i.e. 9 unit/g activity value equals to 1 A590/mg activity value.

Table 10: AC1 activity in grain samples measured by both colorimetric and unit assays

	AC1 activity		Conversion rate
Sample ID	**unit/g**	**A590/mg**	**unit/g : A590/mg**
1	134.59	21.29	6
2	128.88	21.42	6
3	161.14	21.32	8
4	160.44	23.02	7
5	99.39	9.95	10
6	173.13	17.44	10
7	134.32	10.40	13
8	113.12	9.92	11
9	291.55	24.93	12
10	165.07	14.42	11
11	59.31	7.30	8
12	48.68	6.89	7
13	48.87	8.48	6
14	458.95	52.03	9
15	72.09	10.24	7
16	70.41	12.68	6
17	68.54	12.60	5
18	73.77	11.92	6
19	81.53	10.87	8
20	72.37	12.25	6
21	68.26	11.42	6
22	66.77	9.92	7
23	87.88	11.76	7
24	263.85	25.66	10
25	208.72	19.14	11
26	169.21	15.38	11
27	57.20	3.96	14
28	618.18	64.95	10
29	569.66	66.54	9
30	170.82	18.54	9
31	164.15	16.44	10
32	172.93	16.43	11
33	181.24	17.20	11

34	180.54	16.89	11
35	176.32	16.14	11
36	194.35	18.00	11
37	188.97	16.88	11
38	149.32	19.83	8
39	131.41	17.76	7
40	130.86	18.12	7
41	152.23	21.30	7
42	144.66	20.38	7
43	145.23	20.90	7
44	129.65	17.20	8
45	133.33	17.56	8
46	140.26	17.82	8
47	307.88	41.38	7
48	300.17	42.42	7
49	338.94	43.90	8
Average			9

From these observations, we conclude that the use of the colorimetric assay (A590/mg) to calculate the number of units (BG unit/g) present in the Carbohydrase AC1 product or in formulated diets is accurate and justified. Table 11 provides an example of converting the colorimetric assay values (A590/g) from formulated diets from a poultry feeding trial to activity units (unit/kg). The activity recovery is equal to converted units relative to the target units.

Table 11. Estimates of BG units in formulated diets

	Target	AC1 Activity in the Diet		
Trial # 86	unit/kg	A590/g	Converted to unit/kg	% Activity recovery
Diet_1	0	0	0	0.00%
Diet_2	0	0	0	0.00%
Diet_3	50	5.27	47.45	94.90%
Diet_4	100	9.06	81.50	81.50%
Diet_5	200	18.63	167.70	83.85%
Diet_6	400	48.88	439.95	109.99%

7. Glucanase colorimetric assay can also detect activities from commercial enzyme products

To demonstrate that the glucanase colorimetric assay described herein is not limited to detecting only Agrivida Carbohydrase AC1, we tested three digestive enzymes (commercially available as human nutritional supplements) using the colorimetric assay protocol. The enzyme content in a capsule of each product was hydrated in 2 mL water, and then was diluted 100-fold before using 50 µl for

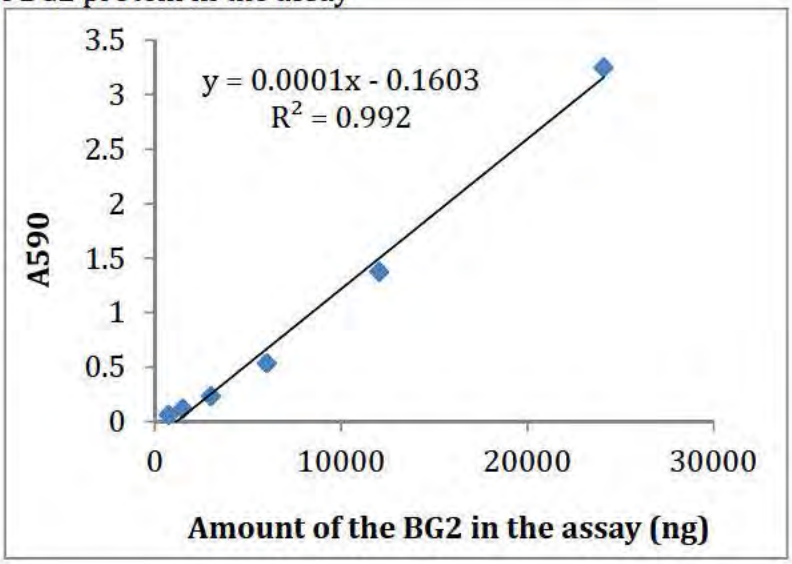
glucanase colorimetric assay [redacted] β-glucanase activity was detected in these products, [redacted] (Table 12).

Table 12. Glucanase colorimetric assay detected activities in commercial enzyme products

Product Brand	Super Digestive Enzymes	Enzymatrix	Digestive Enzymes Ultra
Manufacturer	GNC	100naturals	Pure encapsulations
Labeled β-glucanase unit (BGU)	N/A	65	20
A590 [redacted]	0.102	2.321	0.517
A590 [redacted]	0.2665	Plateaued	1.1035

The glucanase colorimetric assay was also applied to a commercial thermostable β-glucanase enzyme (BG2, Sigma, catalog # G8673). A590 absorbance of BG2 correlates well with the amount of enzyme in the assay as shown in Figure 4.

Figure 4. Correlation between colorimetric assay activity (A590) and the amount of BG2 protein in the assay



10.2 Appendix 2. Carbohydrase AC1 Colorimetric Assay Protocol

1. Required Materials and Equipment

1.1. Apparatus

1.1.1. Roller mill (Roskamp Champion, Model:TP650-9)
1.1.2. Knife mill with 1mm screen (Retsch, Model #SM100)
1.1.3. IKA® Tube Mill 100 Control (IKA, Cat# 0020007145)
1.1.4. Temperature control shaker: New Brunswick, Model: Innova 43
1.1.5. Eppen dorf benchtop clinical centrifuge Model 5810 or equivalent
1.1.6. Laboratory gloves
1.1.7. Pipette tips (Fisherbrand, Cat #: 1-20 µl: 02-707-420 1-100 µl: 02-707-420; Rainin: RT-L1200)
Calibration date:_____ Expiration Date:_____
1.1.8. Pipetman (10-300uL multichannel, 100-1200ul multichannel, 20µl, 100µl and 200µl)
Calibration Date: _____ Expiration Date:_____
1.1.9. 4.5 ml TallPrep Tubes (MP Biomedicals, Cat# 116970000)
1.1.10. 50mL Reagent Reservoirs (Corning- Costar, Cat# 4870)
1.1.11. 96-well Plate Covers (Daigger Lab and Equipment Supply, Cat#100-Ther-PLT)
1.1.12. 96 well block, 2ml (Costar, Cat#3960)
1.1.13. Flat bottom 96 well plate (Costar, Cat #: 9017)
1.1.14. Water bath
Manufacturer: Benchmark
Model number: B2100-12
Serial number: MBG6120U-148
1.1.15 1L glass bottles
1.1.16 500ml glass bottles
1.1.17 Aluminum foil
1.1.18 Timer
1.1.19 Bench top centrifuges
Manufacturer: Spectrafuge
Model number: C2400-B
Serial number: D812826 and D812827
1.1.20 Permanent markers for labeling
1.1.21 Vortex mixer
Manufacturer: Scientific Industries
Model: G-560
Serial number: 2-319492
1.1.22 Metal spatula
1.1.23 pH 4.0 Calibration Solution (OAKTON Instruments, Cat #: 00654-00)
1.1.24 pH 7.0 Calibration Solution (OAKTON Instruments, Cat#: 00654-04)
1.1.25 pH 10.0 Calibration Solution, OAKTON Instruments, Cat# 00654-00)
1.1.26 Balance
Manufacturer: Mettler
Model number: PM4000
Serial number: F80835
1.1.27 Micro plate reader
Manufacturer: Bio-Tek
Model number: Synergy HT

Serial number: 191338

1.1.28 pH meter

Manufacturer: Thermo Scientific
Model number: Orion 3 Star
Serial number: B10839

1.1.29 Falcon 50 mL conical centrifuge tube (BD Falcon, catalog #: 452070)

1.1.30 Micro centrifuge tubes, 2.0 mL (VWR Scientific, catalog# 89000 -028)

1.1.31 PETG Flasks, 250 mL (VWR, Cat# 4112-0250)

1.2 Reagents

1.2.3.2 Adjust volume to 1L with deionized water

1.2.4 Beta-Glucazyme tablets (Megazyme, Cat# T-BGZ1000)

2 Experimental Procedures

2.1 Sample Preparation, Protein Extraction and Extract Dilution

2.1.1 Sample Preparation

2.1.1.1 Grind negative corn seeds for LOD detection in IKA Tube Mill, 23,000 rpm for 1 minute, which produces 94% product with <0.6 mm grind size

2.1.1.2 Grind corn grains and feed in the Knife mill to less than 1.0 mm grind size for protein extraction and activity test

2.1.2 Protein extraction

2.1.2.1 Extract protein by adding 5 volumes Extraction Buffer which was pre-warmed [redacted] (e.g. 10 mL to 2 gram flour or ground feed) in sample containers. Vortex the containers vigorously for 30 seconds, and then shake the containers [redacted], 250 rpm for 1 hour. After one hour extraction, vortex container for 10 seconds before centrifuge 1.5mL of the aqueous extraction at 16,000 g for 10 min. The supernatant is saved for enzyme activity colorimetric assay

2.1.2.2 Purified protein as calibrator: GraINzyme® Carbohydrase AC1 protein was purified from AC1-expressing corn grains, and was stored in [redacted] at -20°C.

2.1.3 Protein dilution for activity assay

2.1.3.1 Protein extract from the negative samples is directly used for activity detection without further dilution

2.1.3.2 Protein extract from AC1-expressing corn product is diluted from 60-fold to 240-fold

2.1.3.3 Protein extract from mash or pellet feed is either not diluted or diluted from 2-fold to 24-fold in the Extraction Buffer

2.1.3.4 The purified AC1 protein is diluted to 5, 10, 20, 31.3, 62.5, 125, 250, 500, 1000, 2000, 3001, and 4000 ng/mL or ppb in the Extraction Buffer

2.2 Colorimetric Activity Assay

[redacted] For the AC1 *product* activity assay, mix 1 tablet of Beta-Glucazyme substrate with 50ul of the protein extract from product or purified protein (calibrator) or buffer blank, and 450ul extraction buffer in a 96 well block. For the AC1 activity assay *in feed*, mix 1 tablet of Beta-Glucazyme substrate with 100ul of the protein extract from feed or buffer blank, and 400ul extraction buffer in a 96 well block. Incubate the block [redacted] for 1 hour. After the incubation, add 1mL of Tris Base to stop the reaction. Centrifugation the mixture at 4000g for 10min, then remove 100ul supernatant to a flat bottom microplate to record A590 using a microplate reader.

3. Results Calculation

3.1 Sample activity calculation

3.1.1 Average A590 readings of the buffer blanks

3.1.2 Subtract the average blank A590 reading from each sample (A590')

3.1.3 Multiply A590' by dilution factors (A590")

A590" = A590' x Extract dilution x Assay dilution

Where:

Extract dilution = 60 to 240 (product); 1 to 24 (feed)

Assay dilution for product = Volume of buffer used for sample extraction (mL)/Volume of protein extract used in enzyme reaction (mL), where 0.05 mL protein extract was used in product activity assay

Assay dilution for feed = Volume of buffer used for sample extraction (mL)/Volume of protein extract used in enzyme reaction (mL), where 0.1 mL protein extract was used in feed activity assay

3.1.4 Divide A590" by the sample dry weight (mg)

3.1.5 AC1 activity of the samples is defined as A590"/mg flour(feed)

3.1.6 Calculate the average and standard deviation

3.2 Positive control activity calculation

3.2.1 Calculate the average of the A590 readings of three positive controls

3.2.2 Subtract the average blank A590 reading from the average positive A590(pcA590)

3.2.3 Divide pcA590 by the amount of positive protein added in the reaction

Where:

0.05mL of 500ng/mL (500 ppb) was added in the reaction, 0.05 x 500 = 25ng = 0.000025mg

3.2.4 Enzyme activity of the positive control (A590/mg protein) equals to pcA590/0.000025

10.3 Appendix 3. Carbohydrase AC1 ELISA Assay Protocol

1. Required Materials and Equipment

1.1. Apparatus

1.1.1. Roller mill (Roskamp Champion, Model:TP650-9)
1.1.2. Knife mill with 1mm screen (Retsch, Model #SM100)
1.1.3. IKA® Tube Mill 100 Control (IKA, Cat# 0020007145)
1.1.4. Temperature control shaker: New Brunswick, Model: Innova 43
1.1.5. Eppendorf benchtop clinical centrifuge Model 5810 or equivalent
1.1.6. Laboratory gloves
1.1.7. Pipette tips (Fisherbrand, Cat #: 1-20 µl: 02-707-420 1-100 µl: 02-707-420; Rainin: RT-L1200)
Calibration date:_____ Expiration Date:_____
1.1.8. Pipetman (10-300uL multichannel, 100-1200ul multichannel, 20µl, 100µl and 200µl)
Calibration Date: _____ Expiration Date:_____
1.1.9. 4.5 ml TallPrep Tubes (MP Biomedicals, Cat# 116970000)
1.1.10. 50mL Reagent Reservoirs (Corning- Costar, Cat# 4870)
1.1.11. 96-well Plate Covers (Daigger Lab and Equipment Supply, Cat#100-Ther-PLT)
1.1.12. 96 well block, 2ml (Costar, Cat#3960)
1.1.13. Flat bottom 96 well plate (Costar, Cat #: 9017)
1.1.14. Water bath
Manufacturer: Benchmark
Model number: B2100-12
Serial number: MBG6120U-148
1.1.151L glass bottles
1.1.16500ml glass bottles
1.1.17Aluminum foil
1.1.18Timer
1.1.19Bench top centrifuges
Manufacturer: Spectrafuge
Model number: C2400-B
Serial number: D812826 and D812827
1.1.20Permanent markers for labeling
1.1.21Vortex mixer
Manufacturer: Scientific Industries
Model: G-560
Serial number: 2-319492
1.1.22Metal spatula
1.1.23pH 4.0 Calibration Solution (OAKTON Instruments, Cat #: 00654-00)
1.1.24pH 7.0 Calibration Solution (OAKTON Instruments, Cat#: 00654-04)
1.1.25pH 10.0 Calibration Solution, OAKTON Instruments, Cat# 00654-00)
1.1.26Balance

Manufacturer: Mettler
Model number: PM4000
Serial number: F80835

1.1.27 Micro plate reader
Manufacturer: Bio-Tek
Model number : Synergy HT
Serial number: 191338

1.1.28 pH meter
Manufacturer: Thermo Scientific
Model number: Orion 3 Star
Serial number: B10839

1.1.29 Falcon 50 mL conical centrifuge tube (BD Falcon, catalog #: 452070)
1.1.30 Micro centrifuge tubes, 2.0 mL (VWR Scientific, catalog# 89000 -028)
1.1.31 PETG Flasks, 250 mL (VWR, Cat# 4112-0250)
1.1.32 Anthos Fluido 2 Microplate Washer (Biochrom, catalog# 80-4000-10)
1.1.33 Titer plate shaker (ThermoFisher Scientific, catalog# 4625Q)

1.2 Reagents

1.2.4 Phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich, catalog# P3563)

Dissolve 1 pack of PBS in 1 L deionized water as protein diluent and plate wash buffer

2. Experimental Procedures

2.1 Sample Preparation, Protein Extraction and Extract Dilution

2.1.1 Sample Preparation

2.1.1.1 Grind negative corn seeds for LOD detection in IKA Tube Mill, 23,000 rpm for 1 minutes, which produces 94% product with <0.6 mm grind size
2.1.1.2 Grind corn grains in the Knife Mill to less than 1.0 mm grind size for feed preparation

2.1.1.3 Grind feed samples in the Knife mill to less than 1.0 mm grind size for protein extraction and activity test

2.1.2 Protein extraction

2.1.2.1 Extract protein by adding 5 volumes Extraction Buffer which is pre-warmed [redacted] (e.g. 100 mL to 20 gram flour or ground feed) in sample containers. Vortex the containers vigorously for 30 seconds, and then shake the containers [redacted] 250 rpm for 1 hour. After one hour extraction, vortex containers for 10 seconds before centrifuge 1.5mL of the aqueous extract at 16,000 g for 10 min. The supernatant is saved for ELISA assay

2.1.3 Protein dilution for ELISA assay

2.1.3.1 Dilute purified AC1 protein to make a standard curve
The purified protein is diluted to 0.12, 0.23, 0.45, 0.90, 1.24, 1.40, 1.60, and 1.80 ng/mL or ppb in PBS buffer

2.1.3.4 Protein extract was diluted with PBS diluent to allow sample ODs to fall on the standard curve

2.2 ELISA Assay

[redacted]

2.2.1 Allow ELISA plate/strips to warm up to ambient temperature before removing the plate/strips from bag with desiccant, it takes about 30 minutes

2.2.2 Calculate the buffer needed for each step, and dispense the buffer into a sample tray 20 minutes before usage. For example, 100 ul of each buffer (i.e. Enzyme Conjugate Buffer, Substrate, and Stop Solution) is required for one sample well at each step. If a test including 80 protein extracts and 16 (2x8) protein standards, at least 10 mL buffer is required. Make sure to keep the Substrate buffer in dark by covering the buffer tray with aluminum foil.

2.2.3 Organize all protein standards, sample extracts, and pipettes so that the next step (2.2.4) can be performed in 5 minutes or less. Multi-channel pipettes are used for all samples and reagents additions.

2.2.4 Add 100ul of non-diluted or diluted protein extract to an ELISA plate. Record the sample IDs.

2.2.5 Add 100 ul of protein standards on the ELISA plate. Mix thoroughly for 30 seconds

2.2.6 Seal wells with a plastic wrap to prevent evaporation and incubate for 15 minutes at ambient temperature

2.2.7 After incubation, remove the plastic wrap. Place the plate on the microplate washer. Perform four washes (300 µL/well). After the wash, aspirate the liquid

2.2.8 Add 100ul Enzyme Conjugate to all wells. Mix thoroughly for 30 seconds

2.2.9 Seal wells with the plastic wrap and incubate for 45 minutes at ambient temperature

2.2.10 Wash the plate as in Step 2.2.7

2.2.11 Add 100ul Substrate to all wells. Mix thoroughly 30 seconds

2.2.12 Seal wells with the plastic wrap and incubate for 15 minutes at ambient temperature

2.2.13 Add 100ul Stop Solution to each well and mix thoroughly by pipette up and down a few times. Using new pipette tips for each row of samples. Need to finish this step for all the samples in as short time as possible (about one minute).

2.2.14 Read absorbance of the plate within 5 minutes after addition of the Stop Solution. Read absorbance at 450 nanometers (A450) with a reference wavelength of 640 nanometers (A640)

3. Results Calculation

3.1 Subtract A640 value of each sample or protein standard from their corresponding A450 value (A450').

3.2 Make average of A450' from two buffer blank (blank_A450')

3.3 Subtract blank_A450' from A450' of each sample or protein standard (A450").

3.4 Make average of A450" from two standards of the same concentration

3.5 Graph the concentration (ng/mL) vs the average A450" of each protein standard on linear scales. Apply a quadratic curve fit.

3.6 Calculate the concentration of each protein extract from the quadratic equation

$$Y = ax^2+bx+c$$

Where,

Y is the A450" of a sample protein extract

a, b, c is numerical coefficent of the quadratic curve

X is the concentration of AC1 detected in the sample extract

3.7 Multiply each concentration (X) by dilutions

3.8 Multiply sample concentration (ng/mL) by the extraction volume to obtain AC1 protein content in the extract (ng)

3.9 Divide sample AC1 content (ng) by the sample dry weight (mg)

3.10 AC1 content in the sample is expressed as ug per gram of dry weight

10.4 Appendix 4. Carbohydrase AC1 Activity Unit Assay Protocol

Introduction: To define activity units for AC1, a two-step approach will be applied.
Step one: enzymatic hydrolysis of barley-β-glucan by AC1 to release reducing sugars such as glucose. When protein extract is appropriately diluted, the initial velocity will be detected within 40min of the reaction. It is important to test units from multiple dilutions of protein extract at 40min for a sample whose activity is unknown. If the activities from different dilutions are comparable, the further test can be done at one or two dilutions.
Step two: detection of reducing sugars from the enzymatic hydrolysis and from a set of glucose standards with BCA reagent
One unit (U) of AC1 activity equals 1 μmol/min glucose reducing equivalents released from 1% barley-β-glucan

1. Required Materials and Equipment

1.1. Apparatus

1.1.1. Roller mill (Roskamp Champion, Model:TP650-9)
1.1.2. Knife mill with 1mm screen (Retsch, Model #SM100)
1.1.3. IKA® Tube Mill 100 Control (IKA, Cat# 0020007145)
1.1.4. Temperature control shaker: New Brunswick, Model: Innova 43
1.1.5. Eppendorf benchtop clinical centrifuge Model 5810 or equivalent
1.1.6. Laboratory gloves
1.1.7. Pipette tips (Fisherbrand, Cat #: 1-20 µl: 02-707-420 1-100 µl: 02-707-420; Rainin: RT-L1200)
Calibration date:_____ Expiration Date:_____
1.1.8. Pipetman (10-300uL multichannel, 100-1200ul multichannel, 20µl, 100µl and 200µl)
Calibration Date: _____ Expiration Date:_____
1.1.9. 4.5 ml TallPrep Tubes (MP Biomedicals, Cat# 116970000)
1.1.10. 50mL Reagent Reservoirs (Corning- Costar, Cat# 4870)
1.1.11. 96-well Plate Covers (Daigger Lab and Equipment Supply, Cat#100-Ther-PLT)
1.1.12. 96 well block, 2ml (Costar, Cat#3960)
1.1.13. Flat bottom 96 well plate (Costar, Cat #: 9017)
1.1.14. Water bath
Manufacturer: Benchmark
Model number: B2100-12
Serial number: MBG6120U-148
1.1.15 1L glass bottles
1.1.16 500ml glass bottles
1.1.17 Aluminum foil
1.1.18 Timer
1.1.19 Bench top centrifuges
Manufacturer: Spectrafuge
Model number: C2400-B

Serial number: D812826 and D812827

1.1.20 Permanent markers for labeling

1.1.21 Vortex mixer
Manufacturer: Scientific Industries
Model: G-560
Serial number: 2-319492

1.1.22 Metal spatula

1.1.23 pH 4.0 Calibration Solution (OAKTON Instruments, Cat #: 00654-00)

1.1.24 pH 7.0 Calibration Solution (OAKTON Instruments, Cat#: 00654-04)

1.1.25 pH 10.0 Calibration Solution, OAKTON Instruments, Cat# 00654-00)

1.1.26 Balance
Manufacturer: Mettler
Model number: PM4000
Serial number: F80835

1.1.27 Micro plate reader
Manufacturer: Bio-Tek
Model number : Synergy HT
Serial number: 191338

1.1.28 pH meter
Manufacturer: Thermo Scientific
Model number: Orion 3 Star
Serial number: B10839

1.1.29 Falcon 50 mL conical centrifuge tube (BD Falcon, catalog #: 452070)

1.1.30 Micro centrifuge tubes, 2.0 mL (VWR Scientific, catalog# 89000 - 028)

1.1.31 PETG Flasks, 250 mL (VWR, Cat# 4112-0250)

1.1.32 Heat Block
Manufaturer: VWR Scientific
Model number: 12621-084 or equivalent
Serial number: 2174

1.2 Reagents

1.2.1.4 Filter buffer and store the buffer at 4C.

1.2.3 Barley-β-glucan, low viscosity (Megazyme, cat # - P-BGBL)

1.2.4 0.5M Hydrochloride (Fisher Scientific, Cat# A142-212)

1.2.4.1 Take 4.132ml of concentrated HCl (12.1N) to a beaker

1.2.4.2 Add 95.868ml of distilled water to adjust volume to 100ml

1.2.5 Pierce BCA Protein Assay Kit

Thermo Scientific, Cat# 23227, Lot# _____

1.2.6 D-glucose standard (Acros Organics, 170080010)

1.2.6.1 100mM glucose stock: 180.2mg/10mL prepared in extraction buffer (100mM sodium phosphate, pH 6.5, 0.01% Tween 20)

1.2.6.2 Dilute 100mM glucose to 10mM, 1mM, 0.8mM, 0.6mM, 0.4mM, 0.2mM, 0.1mM, and 0.05mM in the extraction buffer

1.2.6.3 Store glucose standard in 500ul aliquots at -20C

Glc stock used for dilution (mM)	Glc stock added (ul)	Extraction Buffer added (ul)	Final Glc (mM)	Final vol (ul)
100	200	1800	10	2000
10	200	1800	1	2000
10	160	1840	0.8	2000
10	120	1880	0.6	2000
10	80	1920	0.4	2000
10	40	1960	0.2	2000
10	20	1980	0.1	2000
1	100	1900	0.05	2000

2 Experimental Procedures

2.1 Mill grain or feed to less than 1mm grind size

2.2 Extract protein from samples by adding 5 volumes Extraction Buffer which is pre-warmed [redacted] (e.g. 100 mL to 20 gram flour or ground feed) in sample containers. Vortex the containers vigorously for 30 seconds, and then shake the containers [redacted], 250 rpm for 1 hour. After one hour extraction, vortex container for 10 seconds before centrifuge 1.5mL of the aqueous extract at 16,000 g for 10 min. The supernatant is saved for activity unit assay

2.3 Protein extract dilution

2.3.1 Dilute the extracts by 40-, 160-, 240-, 320 or 360- fold as needed (feed sample might not need dilution or dilution at 2- to 10- fold)

2.3.2 Dilute purified protein 100-fold as a positive control for assay validation

2.3.2.1 Purified AC1 protein (200,000ppb) was stored in 50mM MES, 150mM sodium chloride, pH6.3 buffer plus 40% glycerol at -20C

2.3.2.2 Make 100-fold dilution to 2000ppb

2.4 Barley-β-glucan digestion by AC1

2.4.1 Turn on a water bath before beginning procedure to allow it to come up

2.4.3 Weigh out Barley-β-glucan based on the number of reactions, e.g. 10 samples, 4 dilutions for each sample will need total 40 reactions. Each reaction needs 5mg substrate, therefore, 40*5=200 mg of Barley-β-glucan is required. Need to weigh out at least 220mg to prevent the loss of substrate solution during pipetting. Dissolve the substrate with the extraction buffer

Substrate must be prepared before the test.

Barley-β-glucan weighed _____ mg
Extraction buffer added _____mL
Extraction buffer (mL) = (0.45 * weighed mg of barley-β-glucan)/5 = 0.09 * weighed mg of barley- β-glucan

2.4.4 Dissolve substrate by incubating in the water bath, occasionally vortexing until the substrate is fully dissolved.

2.4.5 Cluster tubes are used for 40min endpoint activity unit assay.

2.4.6 Test up to 9 samples, a negative control, and two positive controls in one cluster tube block.

2.4.6.1 In the block of cluster tubes : dispense 450ul of the substrate into tubes of A2 to D12. Record the name of samples and their dilutions which will be dispensed into the corresponding well in Table 1. These rows will serve as the reaction.

2.4.6.2 Add 450ul of the extraction buffer (no substrate added) to each control tubes from rows E2 to H12 corresponding to each sample tube. This will serve as blank to correct protein content detected by BCA method for each reaction. Record the name of sample in the corresponding tube on Table 1

2.4.7 Add 50ul of diluted sample extract including the negative control to each blank tube first, E2 to H12 excluding E12 and F12.

2.4.8 Add 50ul of diluted protein extract including the negative control to each tube containing substrate (reaction), A2 to D12 excluding A12 and B12.

2.4.9 Add 50ul of 2000ppb purified protein into E12 and F12, then A12 and B12

2.4.10 Cover the tubes with Corning™ Storage Mat III, use the Corning Storage Mat Applicator to seal tubes tightly. Shake the plate at a low speed to mix well.

2.4.11 Place the block in the water bath. Add a heavy metal on the top of the cluster tube to prevent mat pop up [redacted] incubation period.

2.4.12 Start timer for 40min enzyme hydrolysis

2.4.13 Pool enough 0.5N HCL to a sample tray

2.4.14 At 40 min of incubation, take the cluster tube block out from the water bath, remove the mat, and add 100ul 0.5N HCL starting from wells A2 to H12 using a multichannel pipette. Pipette up and down three times. Keep the block on ice.

Table 1 (Deep-well plate)

	1	2	3	4	5	6	7	8	9	10	11	12	Dilutions	
A		Sample_1									Neg. Ctr	Pos. Ctr	160	
B		Sample_1									Neg. Ctr	Pos. Ctr	240	
C		Sample_1									Neg. Ctr		320	
D		Sample_1									Neg. Ctr		360	Reaction
E		Sample_1									Neg. Ctr	Pos. Ctr	160	
F		Sample_1									Neg. Ctr	Pos. Ctr	240	
G		Sample_1									Neg. Ctr		320	
H		Sample_1									Neg. Ctr		360	Blank

2.5 Using BCA method to quantify glucose reducing equivalents

2.5.1 Prepare BCA reagent: mixing reagent A with reagent B by 50:1 (i.e. 50ml reagent A mixed with 1ml reagent B)

2.5.2 In a microplate, to make a glucose standard curve, dispense 75ul of the extraction buffer in the first well of column 1 (A1), 75ul of each glucose standard in the remaining wells of column 1 (one set of glucose standards). Refer to Table 2.

2.5.3 To detect reducing sugars in sample reactions, add 50ul of extraction buffer to the plate (A2 to H12) using multichannel pipette, and then remove 25ul of each reaction and blank from the tubes on Table 1 to the microplate, starting from A2, until all reactions and blanks were added to the microplate. Record the sample ID in each well in Table 2.

2.5.4 Add 175ul BCA reagent to each well with samples or standards using a multichannel pipette, pipetted up and down to mix.

2.5.5 Place the plastic sealed plate to [redacted] heat block for 10 minutes

2.5.6 After 10 minutes incubation, cool the plate on ice for 10 minutes

2.5.7 Spin the plate for 5 minutes to bring down the condensate

2.5.8 Record absorbance at 560nm (A560) for all the samples and controls in the microplate

Reaction/Ctr (ul)	25
Extraction buffer (ul)	50
BCA (ul)	175
Total (ul)	250

Glucose Std (ul)	75
BCA (ul)	175
Total (ul)	250

Table 2 Flat-bottom microplate sample layout (BCA test)

	1	2	3	4	5	6	7	8	9	10	11	12	Dilutions	
A	Ext. buffer	Sample_1									Neg. Ctr	Pos. Ctr	160	
B	0.05	Sample_1									Neg. Ctr	Pos. Ctr	240	
C	0.1	Sample_1									Neg. Ctr		320	
D	0.2	Sample_1									Neg. Ctr		360	Reaction
E	0.4	Sample_1									Neg. Ctr	Pos. Ctr	160	
F	0.6	Sample_1									Neg. Ctr	Pos. Ctr	240	
G	0.8	Sample_1									Neg. Ctr		320	
H		Sample_1									Neg. Ctr		360	Blank

3. Activity unit calculation

One unit (U) of AC1 activity equals 1 µmol/min glucose reducing equivalents released from 1% Barley-β-glucan [redacted], pH 6.5 using the BCA method.

3.1 Glucose standard curve:

3.1.1 Correct absorbance glucose standard (A560) by subtracting the absorbance values from the reagent blank (Column 1: A1) for each of the standards

3.1.2 Plot the absorbance at 560nm versus the concentrations of glucose

3.1.3 Calculate the "best fit" line through the dataset using linear regression.

$Y = aX + b$

Where:

Y is the average corrected absorbance for each glucose concentration standard

X is concentration of each glucose standard

a is the slope

b is the intercept

3.1.4 Determine glucose reducing equivalents in each AC1/barley-β-glucan reaction:

3.1.4.1 Subtract the absorbance at 560nm of the sample blank from the absorbance of each corresponding sample reaction

3.1.4.2 Use the regression equation from the glucose standard to calculate the glucose content (A, umol) in the sample

3.1.4.3 Divide the amount of glucose (umol) released from barley-β-glucan at 40min reaction by 40 to determine the amount of reducing units produced per minute.

A/40 umol/min

Where:

A is the amount of glucose (umol) released from substrate at 40min

Unit = Dilution x (A/40)/gram of flour used for protein extraction

Where

Dilution = E/50 * D * 1 * 24

	Volume (ul)	**Dilution**
Sample was extracted in E ul buffer	E	
Protein extract was diluted by D-fold		**D**
50 ul of the protein extract was added to the reaction with Barley-beta-glucan (final vol. 500ul)	50	**=E/50**
Total volume of hydrolysate (ul)	500	
500ul hydrolysate were mixed with 100ul acid (600ul)	500	**1**
Total mix with acid (600ul)	600	
25 ul of the above reaction (600ul) was added to the BCA test (final 250ul)	25	**24**

3.1.5 The final sample activity will be adjusted by subtracting the activity units of the negative control (protein extracts from the non-AC1-expressing grains) at the same dilution

3.1.6 Validate the assay using positive controls

3.1.6.1 Subtract the blank absorbance at 560nm (average absorbance of E12 and F12)from the absorbance of each reaction test (A12 or B12)

3.1.6.2 Use the regression equation from the glucose standard to calculate the glucose content (umol)

3.1.6.3 Divide the amount of glucose (umol) released from barley-β-glucan at 40 minutes reaction by 40 to determine the amount of reducing units produced per minute.

A/40 umol/minute

Where:

A is the amount of glucose (umol) released from substrate at 40 minutes by purified protein

Unit = Dilution x (A/40)/mg of protein in the assay

Where
Dilution = 24
mg of protein in the assay = 2000*(50/1000)/1000000
where
2000ng/ml was the concentration of the positive control used for the test
50ul of 2000ng/ml positive control was included in the reaction and blank

	Volume (ul)	**Dilution**
50 ul of positive control was added to the reaction with Barley-beta-glucan (final vol. 500ul)	50	
Total volume of hydrolysate (ul)	500	
500ul hydrolysate were mixed with 100ul acid (600ul)	500	
Total mix with acid (600ul)	600	
25 ul of the above reaction (600ul) was added to the BCA test (final 250ul)	25	**600/25 =24**