MULTI-ENZYME SYSTEM FOR FEEDS CONTAINING CASSAVA

Background of the Invention

[0001] The invention relates generally to enzymes for treating animal feeds and, more specifically, to an enzyme system for improving the bioavailability of nutrients from animal feeds containing cassava.

[0002] Cassava is the staple food of at least 500 million people and is the third most important food source in the tropics after rice and corn. It originated in Brazil and perhaps also in Central America and was introduced into Africa many hundreds of years ago. It is a hardy, drought resistant crop that provides acceptable yields on low-fertility soils and is of great importance for subsistence farmers throughout the tropics. According to a report by International Food Policy Research Institute (IFPRI), cassava was the most consumed crop in developing countries between 1983 and 1996, accounting for about 37% of the total roots and tubers consumed as food in these countries.[1] It is projected that Asia will consume an additional 79 million metric tons of roots and tubers by 2020, and the demand for cassava as feed will outpace demand as food.[1]

[0003] Thailand is the third largest producer of cassava after Nigeria and Brazil in 2004, and one of the largest exporter of cassava, amounting to 2 million metric tons to regional and developed countries in the world in 2004 [2]. The Thai Tapioca Trade Association (TTPA, http://www.ttta-tapioca.org/crop.php), which was established in 1963, is one of the many organizations in Thailand promoting the use of cassava as feed and food through education and research.

[0004] Cassava can be applied to feed in three forms either as chips, pellets or meal.[3,4] The production of cassava chips involves two steps, i.e. chippings and sun drying. Fresh cassava roots are fed into the hopper of a chipping machine, to produce about 3-cm diameter size chips. The common practice in Thailand is to chip the roots early in the morning and leave them to dry over 2-3 days to 12-14% moisture content. In the production of pellets, after removal of sand and other impurities, the dry chips are ground in a hammer mill. Cassava particles together with steam are forced through holes in the die. The compressed material emerges hot from the other side of the die; after cooling the strands are cut to length to produce pellets. The pellet diameter ranges from 5 to 6 mm and the length from 15 to 20 mm. Cassava meal on the other hand is the powdered residue of the chips and roots after

processing to extract edible starch. It is generally inferior in quality to chips, pellets and broken roots, and has lower starch content and usually contains more sand.

[0005] With the increase in demand of corn for ethanol production, resulting in almost 70% increase in cost in early 2007,[5] feedmills are under financial pressure to look towards alternative feed ingredients for energy and protein. Agricultural by-products such as cassava pellets, distillers dried grains with solubles (DDGS), faba beans (*Vicia faba*) and sorghum have been evaluated as alternative feed ingredients. The high content and digestibility of starch in cassava makes it an ideal replacement for corn. Scientific studies have confirmed that broilers can be fed with cassava (containing up to 40 mg total cyanide/kg at 500 g/kg) without any adverse effects,[6] and that pigs can be exposed to potassium cyanide at 2 mg/kg body weight without deleterious effect.[7] As the limit of inclusion very much depends on the cynogenic potential of cassava, which is a measure of the action of linamarase on linamarin, cassava pellets and meal are now produced by drying processes to reduce the hydrocyanic acid content.[8]

Summary of the Invention

[0006] Cassava starch contains less amylose than corn.[9] The object of the present invention is to improve the digestibility of cassava by a combination of α-amylase, glucoamylase and pullulanase to hydrolyze the highly branched amylopectin. Initial screening using single enzymes shows that glucoamylase has the highest effect on the amount of reducing sugar released from cassava, followed by α-amylase. The combination of three starch-hydrolyzing enzymes was later shown to release the highest amount of reducing sugar as compared with commercial enzymes for corn-soybean meal diets. We have now formulated a multi-enzyme product that can be used specifically to supplement feeds containing cassava.

Brief Description of the Drawings

[0007] Fig. 1 is a chart of the composition of cassava meal and pellets.

[0008] Fig. 2 is a graph of the effect of single enzyme on starch digestibility of cassava pellets.

[0009] Fig. 3 is a graph of the effect of multi-enzyme products on the release of reducing sugar from cassava meal; each value is the mean of three determinations with error bars as standard deviation.

[0010] Fig. 4 is a graph of the effect of the new product formulation on reducing sugar released from cassava meal; each value is the mean of three separate determinations with error bars as the standard deviation; the specifications of α-amylase, glucoamylase and pullulanase in u/g are indicated below the graph.

Description of Preferred Embodiments

[0011] Materials and Methods

[0012] Proximate analysis of cassava meal and pellet

[0013] Samples of cassava meal and pellet were received from a commercial feedmill in Philippines and their proximate compositions were determined.[10]

[0014] Enzyme Assays

[0015] The activity of the enzyme raw materials was determined using the specific substrates listed in Table 1. Activity of α-amylase, protease, glucoamylase and pullulanase is defined as the absorbance of the hydrolysed substrates under specific incubation conditions. For cellulase and xylanase, the activity was defined as the amount of reducing sugar determined by the Nelson-Somogyi's Reducing Sugar Assay [11]. In each analysis, enzyme activity was recovered from the product in duplicate by extraction with the medium as prescribed in the methods. Each extract was then assayed in duplicate for the respective enzyme activity.

[0016] Table 1. Methods used for determination of various enzyme activities.

Enzyme	Subtsrates (Vendor)	Reference
α-Amylase	Phadebas® Amylase Tablets (Magle Life Sciences)	
Cellulase	Cellulase (Sigma Chemical Co., Inc)	Nelson Somogyi' reducing sugar assay [11]
Protease	Azo-casein (Megazyme Megazyme International Ireland Ltd, Ireland)	
Xylanase	Xylan (Nelson-Somogyi' Reducing Sugar Assay)	Nelson Somogyi' reducing sugar assay [11]
Glucoamylase	Phadebas® Amylase Tablets (Magle Life Sciences)	
Pullulanase	Pullulan (Sigma Chemical Co., Inc)	Food Cehmicals Codex Appendix V [12]

[0017] Since both glucoamylase and α-amylase are traditionally assayed using starch of oligosaccharides as substrates, an indirect assay method was developed to differentiate these two activities in the new formulated product. α-Amylase and glucoamylase activities were first determined in the respective enzyme raw materials using Phadebas® Amylase Tablets,

which is based on color formation from a derivatized starch upon enzymatic hydrolysis. The total amylase activity in the new formulated product was then determined using the same method. Glucoamaylase activity and α-amylase activity can then be calculated after subtracting the total amylase activity by each of the theoretical activities in the product.

[0018] Preparation of Multi-enzyme products

[0019] Laboratory samples of the new formulated product were prepared by manual mixing the appropriate amount of commercial enzyme raw materials listed in Table 1 with carriers and miscellaneous ingredients to provide the desired enzyme activity profile. KEMZYME V and C are existing products of Kemin Industires (Asia) Pte Ltd (Table 2).

[0020] Table 2. Composition (u/g) of multi-enzyme products used in this study

Activity	KEMZYME		
	V Dry	C Dry	TOP Dry
α-amylase	270	450	180
Glucoamylase	-	-	2.5
Pullulanase	-	-	250
Protease	900	1,800	1,000
Cellulase	2,500	5,000	2,500
Xylanase	1,875	2,500	1,100

[0021] *In vitro* reducing sugar release analysis

[0022] Cassava meal or pellet was first ground by a coffee mixer and passed through 1mm mesh sieve. A total of 10.0g of cassava meal or pellets were weighed into a 250 ml conical flask for each treatment. Each treatment was made up to 100 ml with deionized water and treated with 10ml of enzyme mixture solution and incubated for 1 hour at 37°C. The reaction was stopped by placing the samples in boiling water bath for 10 minutes. The supernatant were then analyzed for reducing sugar using Nelson-Somogyi's Reducing Sugar Assay [11]. Each treatment was carried out in triplicate and the means were tested by One-Way ANOVA using the Prism software (GraphPad Inc. USA).

[0023] Results and Discussion

[0024] Proximate analysis

[0025] Proximate analysis shows that both cassava meal and pellet contained more than 80% carbohydrates (Fig. 1), about half of which is starch. Starch content in cassava samples is largely dependant on the cultivar, growth conditions and age [13], which explains why the

amount of starch in cassava meal and pellets are lower than the reported value of 60%. [14] The analysis also shows that cassava contained significant amount of neutral detergent fiber (NDF) and acidic detergent soluble fiber (ADF), both of which are known to have a negative effect on growth performance in broilers and pigs.[15] What is most noticeable from the analysis is the absence of hydrocyanic acid, a strong indication of the extensive processing to which the cassava meal and pellet were subjected.

[0026] Effect of single enzyme on cassava

[0027] A total of six commercial enzyme raw materials were screened for their effect on the release of reducing sugar from cassava pellet, which is expected to have a lower starch digestibility due to high ADF (Fig. 1). Three of the enzymes in this screening experiment are known to hydrolyze the different glucosidic bonds in starch.[16] (Table 3).

[0028] Table 3. Starch-hydrolyzing enzymes used in the screening of enzymes for cassava

Enzyme	E.C. No	Action
α-amylase	3.2.1.1	Endohydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units
Glucoamylase	3.2.1.3	Hydrolysis of terminal 1,4-linked α-D-glucose residues successively from non-reducing ends of the chains with release of β-D-glucose
Pullulanase	3.2.1.41	Hydrolysis of (1-6)-alpha-D-glucosidic linkages in pullulan and in amylopectin and glycogen, and the α- and β-limit dextrins of amylopectin and glycogen

[0029] *In vitro* reducing sugar release test showed that glucoamylase (E.C. 3.2.1.3) is the most effective enzyme, followed by α-amylase (E.C.3.2.1.1) and xylanase (Fig. 2). This also implies that the exo-hydrolysis of starch by glucoamylase is the rate-limiting step of this *in vitro* reaction. As both cassava meal and pellet have over 40% of starch (Fig. 2), it is not unexpected that both glucoamylase and α-amylase released the highest amount of reducing sugar. Since the number of α-1,6 glucosidic bonds in amylopectin is much smaller than that of α-1,4 glucosidic bonds, the effect of pullulanase on reducing sugar released from cassava is expected to be proportionally lower (Fig. 2).

[0030] Development of the new product

[0031] Since most commercial feedmills will add cassava to partially replace corn in corn soybean meal diets, the multi-enzyme product for cassava should still be based on one designed for corn soybean meal diet. The results presented in Figs. 3 and 4 illustrate that the current enzyme products for corn soybean meals do have a significant effect on reducing sugar from cassava meal, and to a lesser extent on cassava chips. This disparity in performance between the two forms of cassava could be attributed to the different amylose/amylopectin ratio, or degree of starch crystallinity, in each of these substrates. Nevertheless, there appears to be a positive correlation between α-amylase and the amount of reducing sugar from cassava meal and chips, indicating that the starch-hydrolyzing enzymes such as glucoamylase and α-amylase should be the dominant enzymes in the new multi-enzyme product for cassava. The new productwas therefore formulated on the KEMZYME V Dry platform with an additional mixture of three starch-hydrolyzing enzymes: glucoamylase, α-amylase and pullulanase.

[0032] The efficacy of the new product was verified against a commercial cassava meal from Vietnam (Fig. 3) and commercial cassava chips from Thailand (Fig. 4). Fig. 3 shows that the reducing sugar released by the new productis significantly higher ($P<0.05$) than a commercial product, Rovabio Excel (Adisseo) by up to 15mg/g. It is also numerically superior to KEMZYME V and C Dry at the same inclusion rate. Fig. 4 shows that the reducing sugar released by the new productis significantly higher ($P<0.001$) than Rovabio Excel by up to 21mg/g and significantly higher ($P<0.05$) than KEMZYME C Dry while numerically superior to KEMZYME V Dry at the same inclusion rate.

According to a previous study, the Apparent Metabolizable Energy (AME) of corn can still be significantly improved ($P<0.05$) by 0.33MJ/kg DM using a multi-enzyme product containing α-amylase, protease, xylanase and cellulase,[17] implying that such multi-enzymes will increase the AME of cassava. However, as observed by Pascual-Reas [18] and Roelof *et al* [19], cassava starch is digested slightly differently from corn starch in the digestive tracts of chickens and pigs. The novelty of this producttherefore lies in the use of a combination of starch-hydrolzing enzymes to hydrolyze both α-1,4 and α-1,6 bonds in amylose and amylopectin. As illustrated in Fig 4, there is a synergistic effect among the three starch-hydrolyzing enzymes: α-amylase, glucoamylase and pullulanase in increasing the amount of reducing sugar from cassava.

[0033] Conclusion

[0034] Despite the long-standing belief that starch in cassava is highly digestible, this study shows that it could be further improved by combination of α-amylase, glucoamylase and pullulanase in a multi-enzyme product. As the cost of corn is expected to rise due to the increased demand of corn for ethanol production, there is a huge economic benefit for livestock owners to partially replace corn by cassava. To date, there is at least one commercial trial confirming the efficacy of this product on diets containing cassava, underlying the pivotal role of enzymes in feeds containing alternative feed ingredients. The successful development of this enzyme product sets the stage for a new series of enzymes targeted against alternative feed ingredients.

[0035] The foregoing description and drawings comprise illustrative embodiments of the present inventions. The foregoing embodiments and the methods described herein may vary based on the ability, experience, and preference of those skilled in the art. Merely listing the steps of the method in a certain order does not constitute any limitation on the order of the steps of the method. The foregoing description and drawings merely explain and illustrate the invention, and the invention is not limited thereto, except insofar as the claims are so limited. Those skilled in the art that have the disclosure before them will be able to make modifications and variations therein without departing from the scope of the invention.

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We claim:

1. A method for improving the nutritional value of an animal feedstuff containing cassava, comprising the step of treating the feedstuff with a combination of α-amylase, glucoamylase and pullalanase.

2. A method as for improving the nutritional value of an animal feedstuff containing agricultural by-products, wherein the feedstuff is selected from the list consisting of cassava chips, cassava pellet, cassava meal and other cassava-containing feedstuffs, comprising the step of treating the feedstuff with a combination of α-amylase, glucoamylase and pullulanase.

3. A method for replacing corn in an animal diet, comprising the steps of:
   (a) selecting an agricultural by-product from the group consisting of cassava chips, cassava pellet, cassava meal and other cassava-containing feedstuffs; and
   (b) treating the by-product with a combination of α-amylase, glucoamylase and pullalanase.

4. The method as defined in claim 1, further comprising carbohydrases selected from the group consisting of beta-glucanase, cellulase, xylanase and pectinase, proteases and phytase..