Phytase Impacts Various Non-Starch Polysaccharidase Activities on Distillers Dried Grains with Solubles

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ABSTRACT

As environmental concerns increase in the U.S., there is a push to go "green". This has fueled the development of ethanol production in the U.S. as a renewable source of energy. In the U.S., corn is used to produce ethanol. Therefore, displacement of corn used in the animal industry to ethanol production has, inevitably caused an increase in the price of corn. Replacing conventional ingredients, such as corn, in monogastric diets with lower cost by-products can be attractive economically. The objective of this study was to determine if exogenous enzymes that free reducing sugars from fiber will increase the dispensability of bound nutrients in the economically favorable by-product of ethanol production, distillers dried grains with solubles (DDGS). Effects of non-starch polysaccharidase (NSPase) inclusion rates and combinations with phytase on the ability to release reducing sugars were investigated. Distillers dried grains with solubles was tested *in vitro* in combination with hemicellulase (0, 0.1, 1, and 10 U/g of DDGS), cellulase (0, 1.5, 15, and 150 U/g of DDGS), xylanase (0, 2.5, 25, and 250 U/g of DDGS), β-glucanase (0, 0.006, 0.06, and 0.6 U/g of DDGS), and phytase (0, 250, 500, and 1,000 FTU/kg of DDGS). Results indicated an inhibitory effect of phytase on the ability of NSPases to release reducing sugars from DDGS (P = 0.0004). A second study was conducted to determine individual enzyme kinetics of NSPase on pure substrates when combined with increasing concentrations of phytase. Cellulase (P = 0.03), xylanase (P = 0.09), and β-glucanase (P = 0.06) combined with increasing concentrations of phytase showed a reduction in the velocity of reducing sugars

release from pure substrate. However, we speculate that due to the inability to reach V_{max}, the Lineweaver-Burk results were inconclusive. The $-1/K_m$ was calculated for all enzymes, and the presence of phytase increased $-1/K_m$ for xylanase (P = 0.006). However, $-1/K_m$ did not change for cellulase (P = 0.62), β-glucanase (P = 0.20), and hemicellulase (P = 0.13). In addition, the $1/V_{max}$ was calculated for all enzymes, and the presence of phytase decreased $1/V_{max}$ for cellulase (P = 0.03) and β-glucanase (P = 0.01). However, the presence of phytase resulted in no change for the $1/V_{max}$ for xylanase (P = 0.81) and hemicellulase (P = 0.14). The slopes of the regression lines for the Lineweaver-Burk plots showed no effect of the presence of phytase for cellulase (P = 0.40) and hemicellulase (P = 0.27). However, the presence of phytase decreased the linear slope for xylanase (P = 0.006), and increased the linear slope for β-glucanase (P = 0.006). In summary, phytase appears to act as an inhibitor of NSPase activity in the *in vitro* digestion on DDGS; however, inconsistent results from the inhibition curves preclude us from determining the type of inhibition.

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Thank you.

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Chapter I

Introduction

Recently, environmental concerns have increased among Americans resulting in a drive to go “green”. This has fueled the development of ethanol production in the U.S. as an alternative fuel source. In the U.S., ethanol is mainly produced from corn. Inevitably, ethanol production has resulted in a rise in the price of corn due to the increased need to produce ethanol, and the ever present need as a feed source. In fact, corn has increased from $2.25 per bushel (25.4 kg) in 2005 to over $7.00 per bushel in June of 2008 (Leibtag, 2008). The production of ethanol as a fuel source from corn removes the starch fraction of the grain, and the solid by-product is distillers dried grains with solubles (DDGS). As ethanol production continues to increase, so will the amount of DDGS produced. In fact, every bushel of corn (25.4 kg) converted to 90% ethanol (10.6 L) also produces 8.2 kg of DDGS (Yoder, 2003; Mosier et al., 2005; Baker and Zahniser, 2006).

As the price of corn continues to escalate, it is important to find alternative feed sources for monogastrics. Distillers dried grains with solubles is higher in non-starch polysaccharides (NSP), crude protein (CP), crude fat (CF), and minerals than is the parent grain. However, monogastrics do not digest feedstuffs high in NSP efficiently. As a result, the metabolizable energy (ME) of DDGS (2,820 kcal/kg) is lower than in corn (3,420 kcal/kg). However, supplementing monogastric diets with exogenous enzymes such as non-starch polyssaccharidases may improve the available energy of DDGS through degrading fiber content and increasing digestibility of other components.

Monogastrics are inefficient at utilizing phosphorus in cereal grains because much of it is bound to phytate. Phytate is the primary form of phosphorus in the seed of cereal grains; where it serves as a retrievable storage unit during development and germination (Raboy, 2003). Phytase is an enzyme that hydrolyzes phosphorus from phytate. However, monogastrics do not produce endogenous phytase, and therefore, have a decreased ability to digest phosphorus and increased fecal phosphorus content. Environmental concerns and increased regulation through amendments to the Clean Water Act (CWA) have put limitations on the amount of phosphorus that can be applied to land in the form of animal manure. As a result, phytase is being added to monogastric diets to reduce fecal phosphorus concentration. The addition of exogenous phytase to monogastric diets increases the bioavailability of phosphorus in grains, and allows for lower inorganic phosphorus inclusion in the diet which translates into lower amounts of phosphorus excreted in manure (Augspurger et al., 2003).

The study conducted in this thesis was performed to determine the most efficient non-starch polysaccharidase and phytase combination in order to increase nutrient availability in DDGS for monogastrics. This was tested through an established *in vitro* digestion procedure that mimics the gastrointestinal system of swine. However, the results were unexpected, and lead us to discover the enzyme kinetics of the individual non-starch polysaccharidase combined with phytase. These results will potentially aid in a more efficient combination of these enzymes in monogastric diets. With more research, these results could influence the next generation of exogenous phytases and non-starch polysaccharidases.

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Chapter II

Review of Literature

Introduction

As environmental concerns increase in the U.S., there is a push to go "green", resulting in an increased need for renewable energy sources, such as ethanol. Ethanol production in the U.S. is escalating. In fact, by 2011 there will be over 40 million metric tons of ethanol produced per year (Wescott, 2007). In order for ethanol production to be sustainable in the U.S., the ethanol industry must find a use for the increasing amounts of by-products it produces. In the U.S. ethanol is mainly produced from corn, thus, the displacement of corn used in the animal industry now being used in the ethanol production has caused the price of corn to increase. In fact, as of June 2008 a bushel of corn (25.4 kg) was over $7.00 as compared to $2.25 in 2005 (Leibtag, 2008). This has forced the animal industry to find alternative sources of feed. Distillers dried grains with solubles (DDGS) is the nutrient rich by-product of ethanol production. However, as a monogastric feed DDGS has some limitations, especially as an energy source. In order for DDGS to be an efficient energy source, enzyme supplementation is necessary. Non-starch polyssaccharidases (NSPase) are enzymes that could improve the energy availability of DDGS for monogastrics. Furthermore, environmental concerns have also put limitations on the amount of phosphorus that can be applied to land in the form of animal manure through amendments to the Clean Water Act (CWA). This has caused many producers to add the phytase enzyme to monogastric diets in order to reduce fecal phosphorus concentrations. Phytase allows

phytate-phosphorus to be utilized by monogastrics; therefore, limiting the amount of inorganic phosphorus needed in the diet, and in turn reducing phosphorus excreted in manure.

Monogastrics

Typical Composition of U.S. Monogastric Diet

Feed is the primary cost of food animal production. Cereal grains comprise the bulk of the monogastric diet. Corn, sorghum, wheat, barley, or triticale are the common energy sources in monogastric diets. Grain prices play an important role on the energy source used in feed. Monogastric diets are generally composed of a mixture of cereal grains, oilseed meal, fats, vitamins, and minerals. However, corn and soybean meal make up the bulk of the feed ingredients in swine and poultry diets in the U.S. Corn is an excellent source of energy. In fact, the metabolizable energy (ME) value of corn is 3,420 kcal/kg for swine and 3,350 kcal/kg for poultry and contains 2.2% crude fiber (NRC, 1994, 1998). Corn averages 8.5% in crude protein (CP), and is low in the dietary essential amino acids lysine and methionine. Corn is also low in calcium (0.02%), total phosphorus (0.28%), and non-phytate phosphorus (0.08%). The major protein source in monogastric diets in the U.S. is soybean meal, which contains 2,230 kcal/kg ME for poultry and 3,180 kcal/kg ME for swine, 44% CP, and 7% crude fiber. Soybean meal is higher than corn in calcium (0.29%), phosphorus (0.27%) and non-phytate phosphorus (0.65%) (NRC, 1994, 1998).

Corn-soybean meal-based diets are not adequate in calcium and moderate in phosphorus. Also, more than half of the phosphorus in cereal grains is organically bound in the form of phytic acid, which is not digested by pigs and poultry. For this reason, phosphorus requirements are generally expressed in non-phytate phosphorus. Growing poultry need 0.45% and growing pigs need 0.40% of non-phytate phosphorus in the diet to thrive. Monogastric diets are typically fortified with sources of highly bioavailable phosphorus in order to meet the non-phytate phosphorus requirements and avoid bulkiness of the diet. Common sources of high bioavailable phosphorus are steamed bone meal, mono- and di-calcium phosphate, deflourinated phosphate, phosphate from ground rock curacao, soft rock phosphate, sodium phosphate, and phosphoric acid (NRC, 1994, 1998). Many of these phosphorus supplements also have a wide range of bioavailability to monogastrics.

Protein sources of animal origin can be added to the diet as a phosphorus supplement. These include milk, blood, meat, and bone by-products. Of these, milk and blood by-products are highly bioavailable in phosphorus (Cromwell et al., 1979; Hew et al., 1982; Coffey and Cromwell, 1993) and meat and bone meal by-products are variable in phosphorus bioavailability. In fact, meat and bone meals have shown to range from 67% compared to other animal sources (Cromwell, 1992) to as high as 90% compared to highly bioavailable sources of inorganic phosphorus (Traylor and Cromwell, 1998). In addition, phosphorus availability from inorganic sources is also highly variable, and typically depends on the source and processing methods (Kornegay and Radcliffe, 1997). Typically, ammonium, calcium, and sodium phosphates are more bioavailable than the other inorganic phosphorus sources such as deflourinated phosphate, phosphate from ground rock curacao, and soft rock phosphate (Chapman et al., 1955; Hays, 1976; Cromwell, 1992).

Corn Composition

The corn kernel is composed of 4 components: the endosperm, pericarp, tip cap, and germ (**Figure 2.1**). The endosperm is the source of energy and protein for the germinating seed. The main energy source within the endosperm is starch, which comprises 71% of the kernel dry weight. The endosperm is 85% of the total kernel. The corn kernel is approximately 8.5% CP (Larkins et al., 1993; Gulati et al., 1996; Yoder and Tolman, 2003). The pericarp is the outer layer covering of the kernel and functions to protect the germinating seed from microorganisms and water leaching. This outer layer is composed of nearly 9% crude fiber, which greatly contributes to the total crude fiber content of the kernel. In fact, 83% of the crude fiber comes from hemicellulose, cellulose, and lignin. Over 50% of the fiber within the kernel is found in the pericarp and germ (Gulati, 1996). The tip cap connects the kernel to the corn cob. The only living portion of the kernel is the germ, which contains genetic information for the germinating seed. The germ also contains some enzymes, vitamins, and minerals available to the growing seed (Yoder and Tolman, 2003). Both phytase and phytic acid are also stored in the germ (O'Dell et al., 1972; Yoshida et al., 1999).

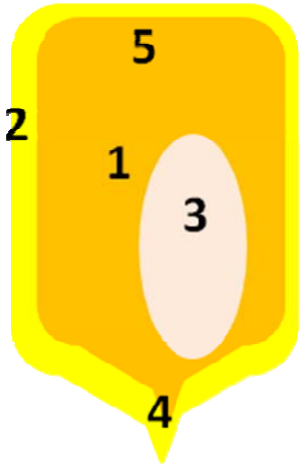


Figure 2.1. Structure of the corn kernel. The endosperm (1) is the primary source of energy and protein for the germinating seed. The pericarp (2) is the outer layer of the kernel protecting the germinating seed. The embryo or germ (3) is the living portion of the kernel containing the genetic information of the growing seed. The tip cap (4) attaches the kernel to the corn cob. The aleurone layer (5) is the outer 1 to 3 layers of cells within the endosperm.

adapted from Yoder and Tolman, 2003

Phytic Acid in Cereal Grains

myo-Inositol is a cyclic alcohol derivative of glucose (**Figure 2.2**) and provides the backbone structure for phytate (**Figure 2.3**) (Loewus and Murthy, 2000). Phytic acid is the common name for myo-Inositol-1,2,3,4,5,6-hexa*kis*phosphate (Raboy, 2003). Phytic acid is the primary form of phosphorus in the seeds of cereal grains; where it serves as a retrievable storage unit during development and germination. In fact, phytic acid represents approximately 75% of the seed's total phosphorus (Raboy, 2003). There are 2 conformational forms of phytic acid, 1ax/5eq and 5ax/1eq. However, the sterically favorable form found in plants is the 1ax/5eq conformation (**Figure 2.4**). This conformation contains 1 axial phosphate and 5 equatorial phosphates. The sterically hindered form 5ax/1eq can easily be stabilized by forming salt complexes with metal cations, collectively referred to as phytin

(Loewus and Murthy, 2000). These complexes result in a reduction of inositol, and bioavailability of phosphorus and essential mineral cation for monogastrics. For this reason, phytate has been linked to many health problems including anemia, rickets, osteoporosis, and bone deformities (Berlyne et al., 1973; Taylor, 1965). Another common structure of phytic acid is in the form of phytate, the hexa*kis*phosphoric acid ester of *myo*-inositol **(Figure 2.5)**.

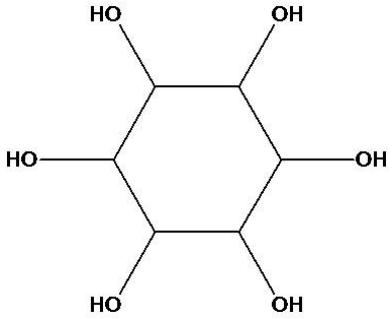


Figure 2.2. *myo*-Inositol. The cyclic alcohol derivative of glucose, which provides the backbone for phytate.

adapted from Leowus and Murthy, 2000

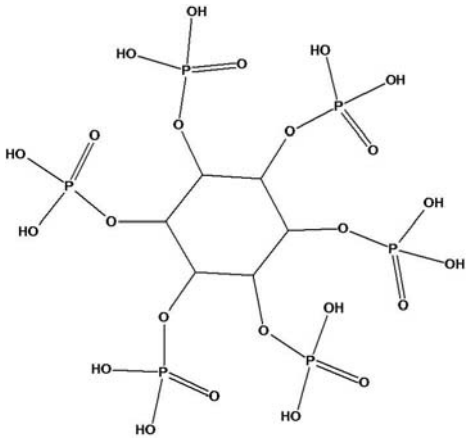


Figure 2.3. Phytic acid. Phytic acid is composed of a *myo*-inositol backbone with 6 phosphate groups.

adapted from Raboy, 2003

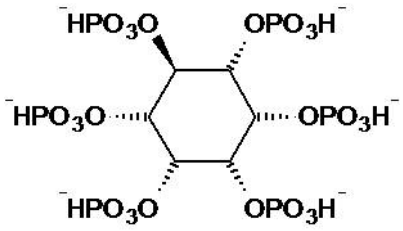


Figure 2.4. Phytate. The sterically favorable form of phytate is 1 axial and 5 equatorial conformation.

adapted from Azevedo and Saiardi, 2006

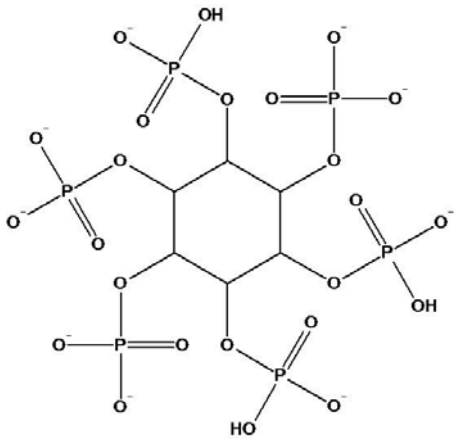


Figure 2.5. Phytate. Phytate is the acid ester form of phytic acid.

adapted frpm Leowus and Murthy, 2000

Synthesis of phytic acid is metabolically unique to plants and takes place in the cisternal endoplasmic reticulum of the cell within the endosperm (Yoshida et al., 1999). In order for phytic acid synthesis to occur, *myo*-inositol must be formed from glucose. *myo*-Inositiol 3-phosphate ($Ins(3)P_1$) is biosynthesized from glucose-6-phosphate by the enzymes hexakinase (EC 2.7.1.1), *myo*-inositol phosphate phosphatase (EC 3.1.3.25), and *myo*-inositol phosphate synthase (EC 5.5.1.4; Drobak, 1992; Loewus and Murthy, 2000). *myo*-Inositiol 3-phosphate **(Figure 2.6)** is converted to phytic acid for phosphorus storage

and homeostasis within the developing seed (Biswas and Biswas, 1996). Currently, there are 2 proposed mechanisms for this conversion **(Figure 2.7)**. This primarily involves sequential phosphorylation of inositol by one or more kinases. The other proposed mechanism involves an inositol derivative as an intermediate that is hydrolyzed to phytic acid (Scott and Loewus, 1986). Post phytic acid synthesis in the cisternal endoplasmic reticulum, it migrates toward the protein bodies in phytin-bearing vesicles. The discharge of vesicular contents results in the accumulation of phytin-rich particles as a globoid in the protein bodies. Phytin is *myo*-inositol hexa*kis*-dihydrogen phosphate calcium magnesium salt form of phytate (**Figure 2.8**).

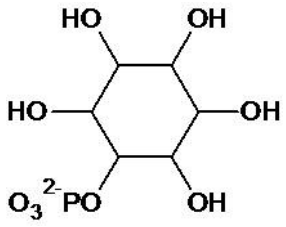


Figure 2.6. *myo*-Inositol 3-phosphate ($Ins(3)P_1$). The precursor to phytic acid within the developing seed.

adapted from Biswas and Biswas, 1996

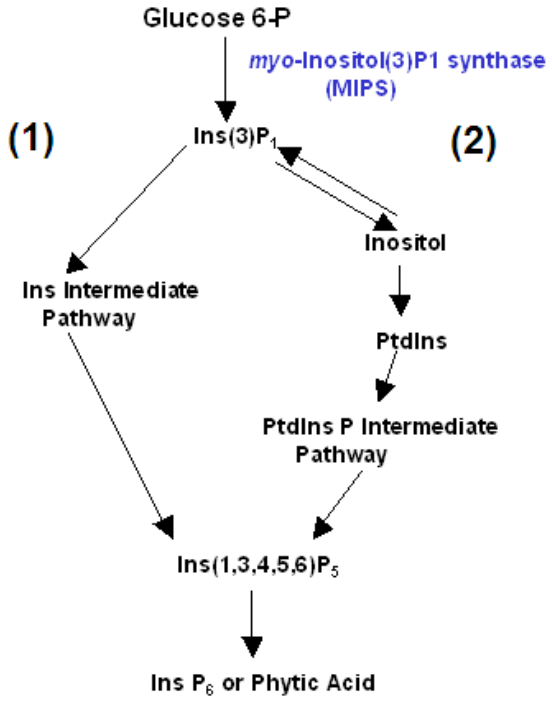


Figure 2.7. Proposed mechanisms for conversion of glucose 6-P to phytic acid. Mechanism 1 involves an inositol derivative intermediate. Mechanism 2 involves sequential phosphorylation of *myo*-inositol.

adapted from Biswas and Biswas, 1996

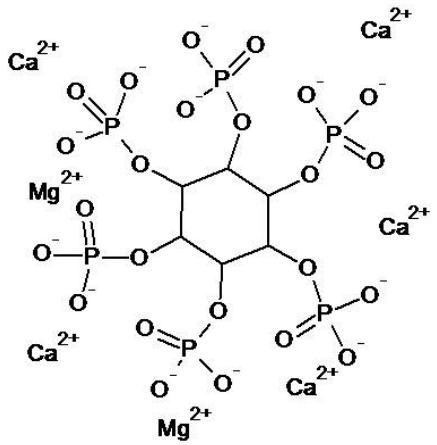


Figure 2.8. Phytin. *myo*-Inositol hexa*kis*-dihydrogen phosphate calcium magnesium salt is a common storage form of phytate in cereal grains.

adapted from Raboy, 2003

Not every cell within the seed stores a form of phytate. In most cereal grains, phytin is found in the protein bodies of the aleurone layer **(Figure 2.1)**. The aleurone layer surrounds the endosperm and is 1 to 3 cell layers thick. However, phytate is not seen in the protein bodies of the single cell layer of the aleurone layer in corn. In fact, 90% of kernel phytate is found within the embryo (i.e., the germ), the thin-walled structure containing the new plant, or the starchy endosperm **(Figure 2.1)**. In addition, the globoid crystals containing phytate are small and non-uniformly distributed in the protoderm and provascular regions of the embryo (O'Dell et al., 1972). Furthermore, phytic acid accumulates only in the cells that remain alive through seed development and germination.

Phytate plays a major role throughout plant development. During the cell division phase, 2 to 4 wk following pollination, the corn kernel begins synthesizing phytin (Earley and De Turk, 1944). It is during the cell expansion phase of the developing kernel that all additional phosphorus is converted to phytic acid. During the seed maturation phase, phytin accumulation occurs approximately simultaneously as the accumulations of lipid, starch, and protein reserves within the endosperm (Greenwood and Bewley, 1984). These reproductive phases are followed by a period of dormancy. Phytate remains stored typically in the form of phytin mixed with several mineral cations during dormancy in preparation for large demands of phosphorus during seed development (Maga, 1982). During the first 24 h of development, there is an increase in inorganic phosphorus and endogenous phytase activity within the endosperm and scutellum of corn seedlings. *myo*-Inositol-hexa*kis*phosphate phosphohydrolase (EC 3.1.3.8) is the endogenous enzyme responsible for phytate degradation within the plant. Simultaneously, there is a decrease in phytate phosphorus (Eastwood and Laidman, 1971; Yoshida et al., 1999). This results in the disassembly of the

phytate salts providing the germinating seed and growing seedling with phosphorus, *myo*-inositol, and mineral cations to be used in support of anabolic metabolism (Dmitrieva and Sobolev, 1984). Besides the major role phytic acid plays in phosphorus homeostasis, it also acts as a natural antioxidant due to the ability to chelate free iron. For example, Fe^{3+}-phytate does not support hydroxyl-radical generation due to the lack of a reactive coordination site (Graf et al., 1987).

In order for the germinating seed to utilize phosphorus in the storage form phytate, it must produce an endogenous phytase. In corn seedlings, phytase activity plateaus between d 5 and 7 post germination. The corn phytase is a unique acidic phytase with an optimum pH of 4.8 at 55°C. Unlike the monomeric phytases from other cereal grains, corn phytase consists of 2 38-kDa subunits. The enzyme accumulates in shoots (mesocotyl plus coleoptiles), which may be related to the primary location of phytate in the germ or embryo. The corn phytase has an apparent K_m of 117 μM and demonstrates a specific activity for 5- and 3-phosphate esters of *myo*-inositol (Laboure et al., 1993).

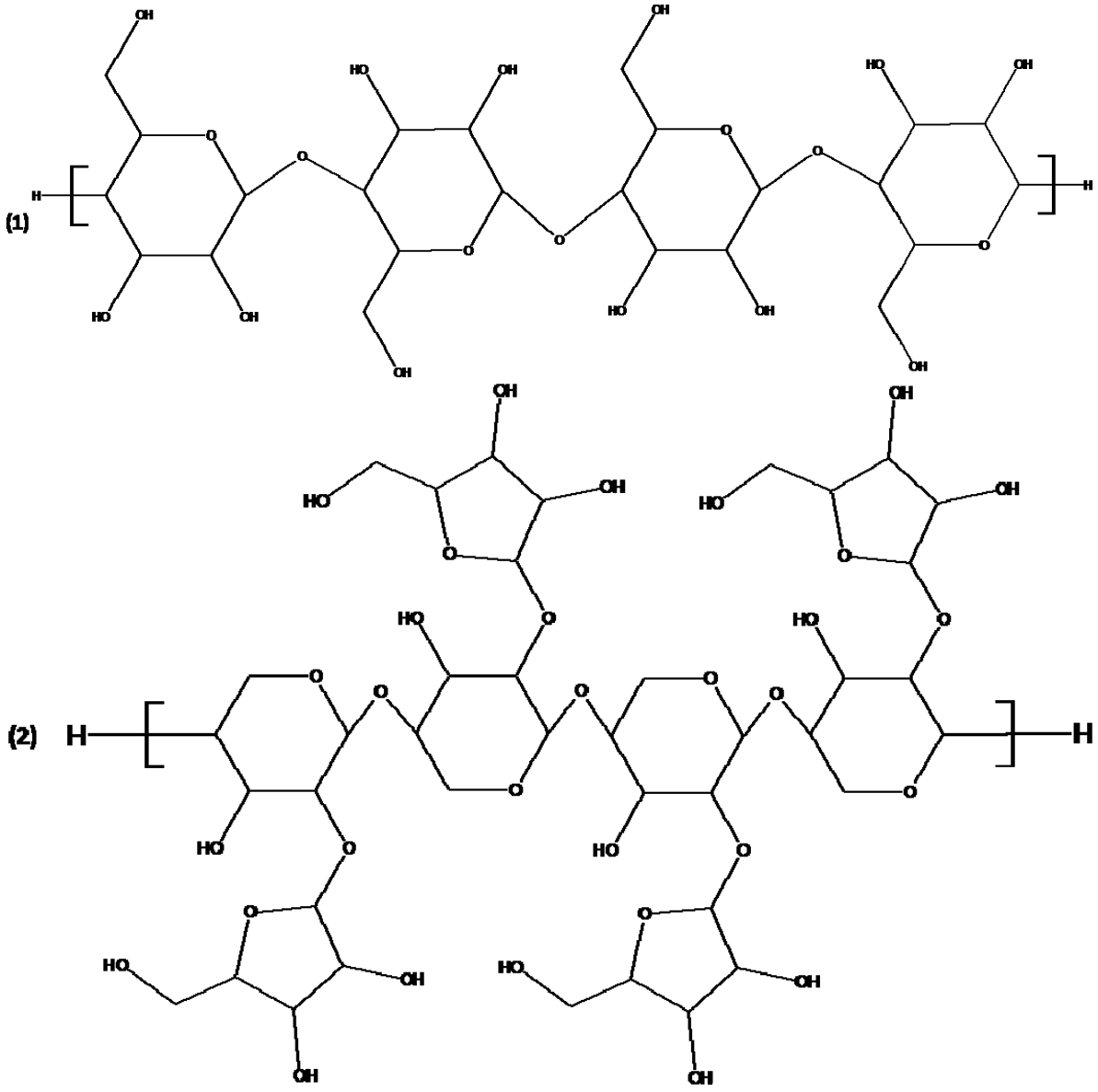
Inositol and inositol phosphates also aid the plant in many metabolic processes. Inositol phosphates are used in the plant during signal-induced hydrolysis to yield diacylglycerol and Ins(1,4,5)P_3. The signals include phytohormones, light induced leaf movements, plant pathogen interactions, gravity, and stomatal closure of guard cells, which inhibits gas exchange among the inner parts of the plant (Drobak, 1993). Inositol phosphates also act as second messengers controlling calcium flux within cells. *myo*-Inositol (1,4,5)P_3 releases calcium from tonoplast vesicles, which contain water, inorganic ions, and enzymes, and increases cytoplasmic calcium concentrations (Schumaker and Sze, 1987; Gilroy et al., 1990). Inositol may also act as a precursor for cell wall biosynthesis (Loewus and Murthy,

2000). The inositol released from the action of endogenous corn phytase on phytate becomes incorporated into the vacuolar membrane of lipids to form phosphoinositide. Then, phosphoinositide is passed to the endoplasmic reticulum membranes, and finally recycled to the plasmalemma (Browse and Somerville, 1991).

Non-Starch Polysaccharides in Cereal Grains

Plant cell walls contain many insoluble polysaccharides. The main polysaccharides are cellulose, arabinoxylans, mixed linked β(1-3)(1-4)-D-glucan which is also known as β-glucan, xyloglucans, rhamnogalacturonans, and aribinogalactans **(Figure 2.9)**. These polysaccharides are composed of pentoses **(Figure 2.10)**, hexoses **(Figure 2.11)**, 6-deoxyhexoses, and uronic acids **(Figure 2.12)**. In the cell wall, cellulose forms a rigid skeleton of microfibrils. The microfibrils of cellulose are embedded in a gel-like matrix composed of the remaining polysaccharides and glycoproteins (Carpita and Gibeaut, 1993). The last main component of the cell wall is lignin **(Figure 2.13)**. Lignin is a branched network of phenylpropane units which anchors the cellulose microfibrils and other matrix polysaccharides to stiffen the cell wall. Lignin continues to be laid down with age. This stiff skeleton-like cell wall prevents physical damage and biochemical degradation. These cell wall complexes are found in cells located in the aleurone layer **(Figure 2.1**; Selvendran, 1984; Iiyama et al., 1994; Theander et al., 1994). The composition of plant cell walls varies among species. Furthermore, compositional variation is seen between tissue types and maturity of the plant. All cereal grain cell walls are composed of these nonstarch polysaccharides: arabinoxylans, which create hemicellulose, cellulose, and β-glucans.

However, there is some variation in the amounts of each nonstarch polysaccharide among cereals (Selvendran, 1984).



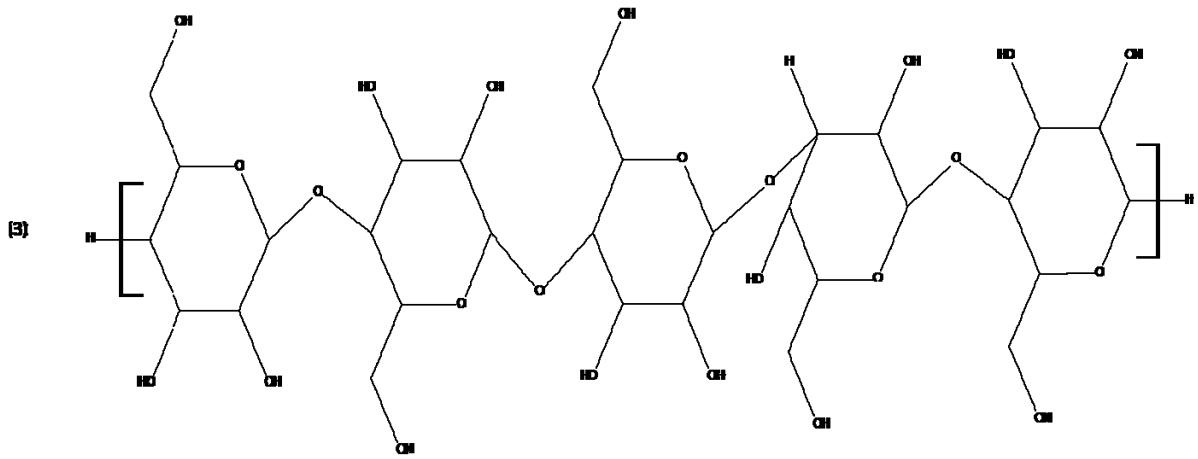


Figure 2.9. Plant cell wall polysaccharides. (1) Cellulose, (2) arabinoxylans, and (3) β-glucan.

adapted from Zamora, 2005

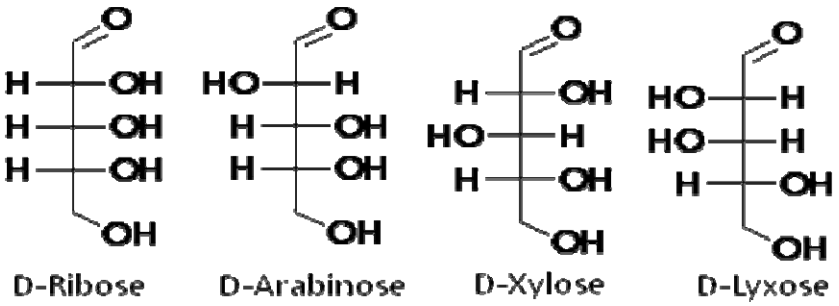


Figure 2.10. Examples of pentoses.

adapted from Bedinghaus and Ockman, 2006

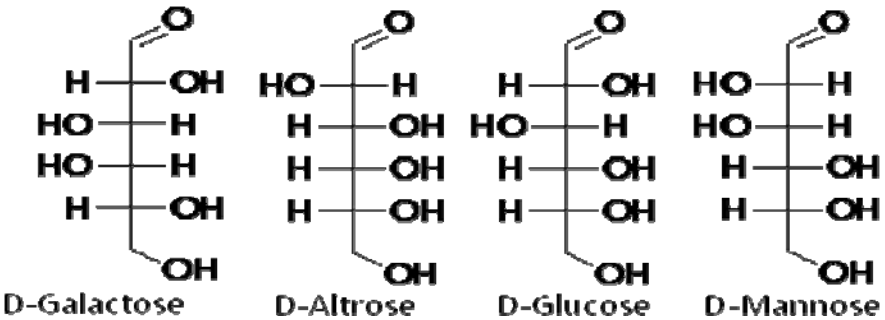


Figure 2.11. Examples of hexoses.

adapted from Bedinghaus and Ockman, 2006

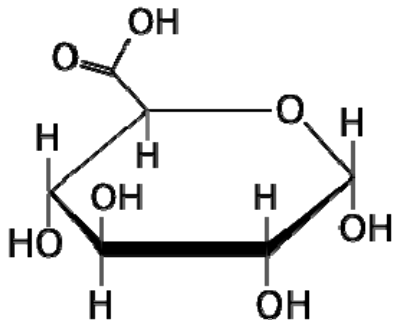


Figure 2.12. β-D-Glucoronic acid is an example of a uronic acid.

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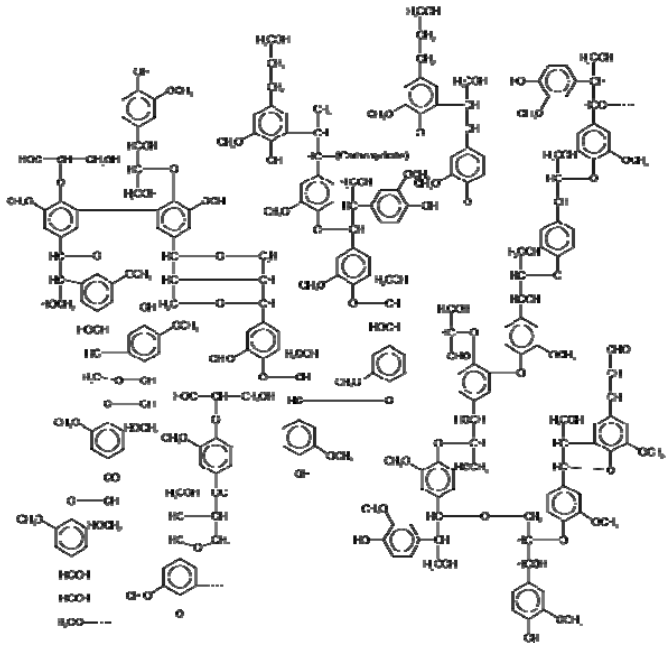

Figure 2.13. Chemical structure of lignin.

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Gastrointestinal System of Monogastrics

The digestive tract of the pig is composed of 5 major sections: the mouth, stomach, small intestine, cecum, and large intestine **(Figure 2.14).** The glandular stomach is

approximately 30% of the total volume of the gastrointestinal tract and secretes HCl from parietal cells and pepsinogens, molecular weight 42.5 kDa, from chief or peptic cells. Pepsinogens are cleaved into pepsins, molecular weight 35 kDa, when gastric pH is below 5.0. These pepsins initiate protein digestion by splitting the interior peptide linkages. Limited mixing also occurs in the stomach. In addition, some microbial activity may occur only if the pig ingests high quantities of feed (Wenk, 2001; Johnson, 2007). The small intestine of the pig is the primary site of digestion and absorption of proteins, starches, lipids, minerals, and vitamins. Enzymes secreted from the exocrine pancreas and bound to the brush border membrane of the small intestine degrade starches, proteins, and lipids into smaller absorbable compounds at a nearly neutral pH. The majority of vitamins and minerals are also absorbed in the small intestine. In fact, approximately 35% of phosphorus is absorbed in the duodenum, 25% in the jejunum, and 40% in the ileum (Wenk, 2001; Johnson, 2007). The cecum is the first 7 to 8% of the 5-m long large intestine in an adult hog. The cecum and large intestine house a vast microbial population which thrives on undigested components. Microbiota populations consist of enterobacteria, anaerobic, coliform, and lactic acid bacterial and yeast populations (Jørgensen and Just, 1988; Anguita et al., 2006). Undigested components consist of dietary fiber, lipids with high melting points, and insoluble proteins. The end products of large intestinal microbial fermentation are mainly short-chain fatty acids, which can contribute 10 to 24% of the total ME for grow-finish swine (Bach Knudsen and Hansen, 1991; Wenk, 2001).

Figure 2.14. Porcine gastrointestinal tract.

The digestive system of a chicken is pictured in **Figure 2.15**. However, anatomical differences from the pig include a crop, gizzard, and paired ceca. The crop is an extension of the esophagus and functions as a storage compartment. Little microbial fermentation is seen in the crop, unless feed is held for extensive periods of time (Stevens and Humes, 1995). The proventriculus is the true stomach secreting both HCl and pepsinogen. Following the proventriculus is the gizzard. The gizzard is a muscular organ used in grinding feed. This evolutionary organ is necessary due to the lack of teeth in avian species. The digesta rate of passage is quicker in poultry than swine. The paired ceca slow the passage rate and allow for microbial fermentation. The absorption that takes place post small intestine can contribute up to 33% to total ME for the bird (Stevens and Humes, 1995; Wenk, 2001).

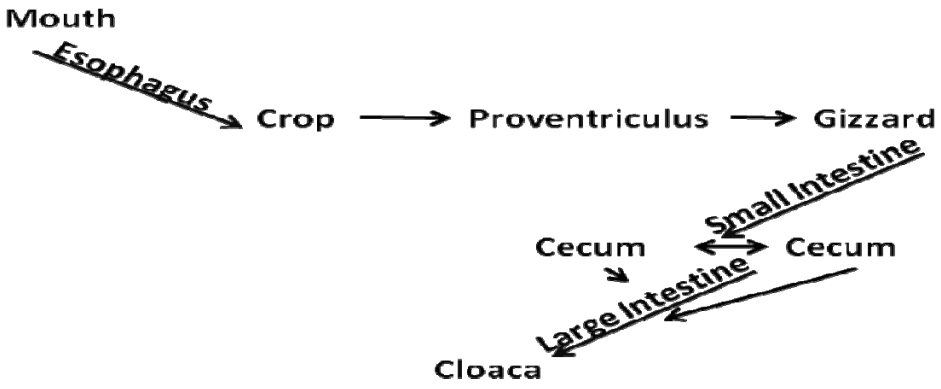

Figure 2.15. Avian gastrointestinal tract.

Limitations of the Monogastric Gastrointestinal System with Regards to Phosphorus

Unlike fungi, plants, and bacteria, vertebrates do not produce phytase. The lack of microbial fermentation prior to the small intestine results in an impaired absorption of many

bound nutrients. As a result, phosphorus bound to phytate is not released before its primary absorption site in the small intestine due to the absence of phytase (Moore and Tyler, 1955a, b). This results in an ineffective utilization of phytate-phosphorus. However, microbial fermentation which occurs in the large intestine results in the release of phosphorus, which is poorly absorbed by the animal. As a result, high levels of phosphorus are found in feces, which can leach into the environment (Wise and Gilburt, 1982; Lantzsch et al., 1988; Pagano et al., 2007). In contrast, microbial populations in the rumen of ruminant animals also produce phytase capable of cleaving the phosphorus from *myo*-inositol, making more phosphorus available for absorption in the true stomach and small intestine (Hill et al., 2008).

Limitations of the Monogastric Gastrointestinal System with Regards to Non-Starch Polysaccharides

Vertebrates also lack the ability to produce NSPase. This, along with the lack of microbial fermentation results in a poor ability to utilize fiber. As a result, high-fiber diets coincide with reductions in foregut and whole tract digestibility of organic matter. High-fiber diets also contain less ME and increase the viscosity of the digesta. When dietary crude fiber exeeds 10 to 15% of the monogastric diet, feed intake is lowered due to reduced palatability and increased bulkiness of the diet (Braude, 1967). Fiber utilization by monogastrics varies considerably depending on the degree of lignification (Forbes and Hamilton, 1952), inclusion rate (Farrell and Johnson, 1970; Just, 1979), processing time and method (Saunders et al., 1969; McNab, 1975), and physical characteristics of the total diet (Schneider and Lucas, 1950; Myer et al., 1975).

Non-starch polysaccharides (NSP) are the primary components of dietary fiber. All components of dietary fiber are resistant to degradation by mammalian enzymes. However, dietary fiber can be degraded with microbial fermentation. Microbial populations found in the rumen of ruminant mammals produce substrate-specific enzymes capable of cleaving the β-linkages of NSP (Macfarlane and Englyst, 1986; Bach Knudsen, 2001). The lack of microbial fermentation prior to the small intestine in the monogastric renders these NSP as an undigestible energy source. However, high-fiber diets cause changes in the microbial populations in the monogastric colon. For example, piglets fed high concentrations of fermentable NSP have higher colibacilli counts (McDonald et al., 1999; Hopwood et al., 2004). Any undigested dietary fiber will be excreted in the feces.

Phosphorus

Environmental Phosphorus Concerns

Phosphorus pollution is of increasing environmental concern. Excess phosphorus that accumulates in the soil can reach waterways through leaching into groundwater or high soil erosion to surface water sources. Eutrophication is caused by an increase in nitrogen or phosphorus in a body of water, leading to an overgrowth of certain algae and plant material, which depletes the oxygen availability to algae, plant and animal populations; followed by decay of algae and plant material. The process of eutrophication decreases the water quality (Koelsch, 2006). Recently, the attention paid to the amount of phosphorus pollution derived from intensive animal agriculture has increased. In fact, over 1 million metric tons of phosphorus derived from animal manure enter the environment in the U.S. each year

(Cromwell and Coffey, 1991; Poulsen, 2000). Due to the amount of phosphorus in animal feces, the U.S. government has made amendments to the CWA. The original CWA was passed in 1972 to prohibit pollutant discharge into U.S. waterways. Over the years, the Environmental Protection Agency (EPA) has identified concentrated animal feeding operations (CAFO) as a source of water pollution. Effective on April 14, 2003, CAFO were subject to National Pollutant Discharge Elimination Systems permit regulations (Koelsch, 2006). This led to the requirement for all CAFO to have a Nutrient Management Plan (NMP) requiring evaluation of the potential nitrogen and phosphorus loss on fields receiving manure, litter or wastewater products. All CAFO were to have and start implementing a NMP by December 31, 2006 (Sheffield and Paschold, 2006). These changes to the CWA have resulted in further limitations to manure spread due to phosphorus content. Prior to the CWA amendments, manure spread was only limited on a nitrogen basis. Plants require less phosphorus than nitrogen for vegetative growth. Therefore, limiting the amount of manure spread based on phosphorus concentration and has resulted in an increased use of phytase in monogastric diets.

Phytase Supplementation

A common misconception is that phytase supplementation to monogastric diets lowers the amount of phosphorus excreted in the feces. However, this is an indirect effect. When the diet is supplemented with phytase, the need for inorganic phosphorus supplementation is decreased. The supplemented phytase hydrolyzes the phytate molecule within the cereal grains of the diet. The released phosphorus from phytate is highly

bioavailable for the animal and can be readily absorbed (Nasi, 1990; Jongbloed et al., 1992; Cromwell et al., 1995). For example, fecal phosphorus excretion was 21.5% lower in grow-finish hogs supplemented with 500 phytase units (FTU)/kg compared to a diet without phytase supplementation that meets the phosphorus NRC (1998) requirements (Harper et al., 1997). It is important to notice that the phytase-supplemented diet contained less supplemental inorganic phosphorus than the control diet. In other words, if a diet is supplemented with phytase and the inclusion of inorganic phosphorus is not lowered, a reduction in fecal phosphorus content cannot be expected.

myo-Inositol-(1,2,3,4,5,6)-hexa*kis*phosphate phosphohydrolase, generically known as phytase (E.C. 3.1.3.8), is a phosphatase that hydrolyzes in a step-wise manner the phosphomonoester bonds of phytic acid to orthophosphate, a series of lower phosphate esters of *myo*-inositol and eventually *myo*-inositol **(Figure 2.16)**. The generic term phytase represents several classes of enzymes with different structures, optimum temperatures and pH, and binding sites to phytate. Phytases can be classified based on the optimum pH for activity as acidic, neutral, and basic. Thus, phytases can be considered as acid, neutral or alkaline phosphatases. It is the acid phytases that are commonly used in the diets of monogastrics (Mullaney and Ullah, 2003). The acidic phytases can be further classified into 3 groups: β-propeller phytase (BPP), purple acid phosphatases (PAP), and histidine acid phosphatase (HAP; Ullah, 1991; Kerovuo, 1998; Hegeman and Grabau, 2001).

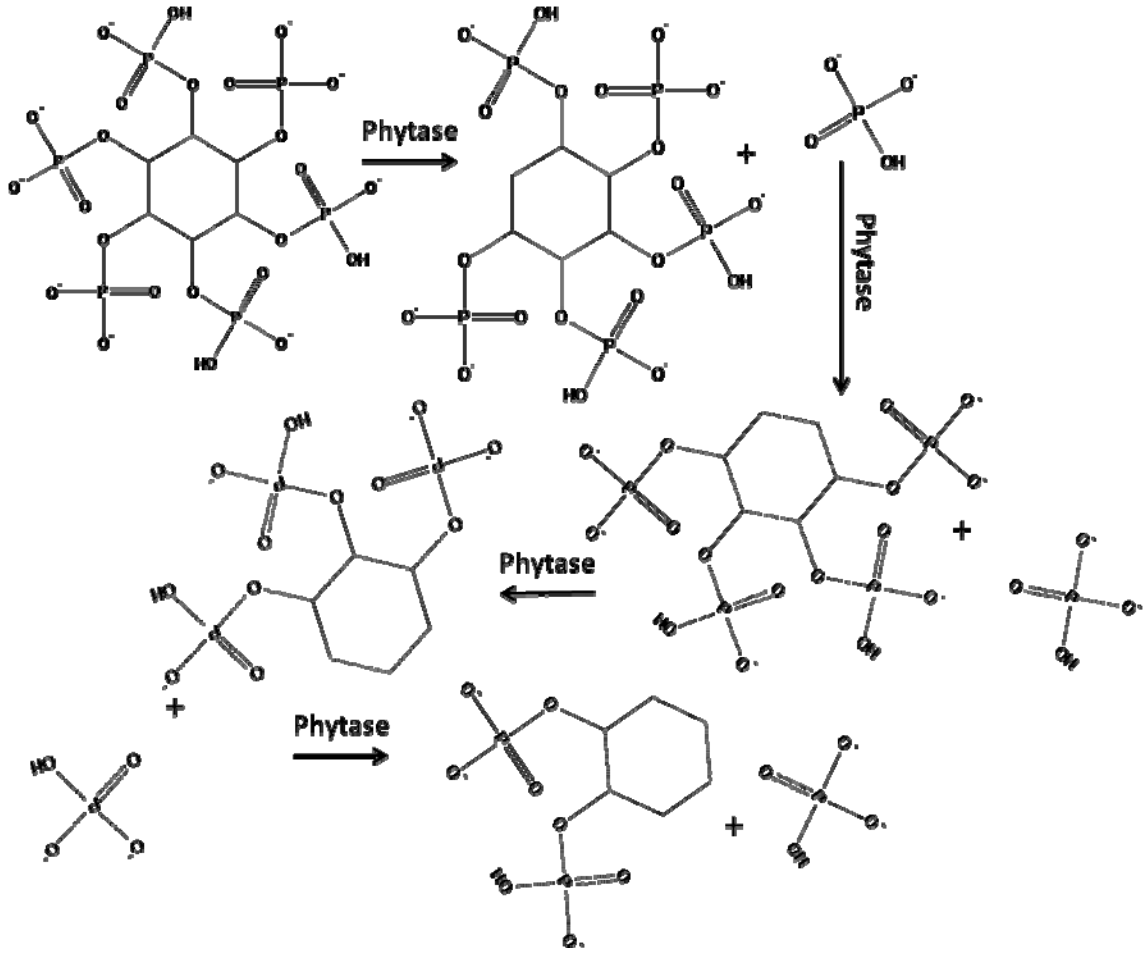

Figure 2.16. Step-wise hydrolysis of phosphate from phytic acid.

adated from Biswas and Biswas, 1996

The BPP (E.C. 3.1.3.8) are a newer class of enzymes with 2 phosphate binding sites. Substrate hyrdrolysis occurs at the "cleavage" and "affinity" sites. β-propeller phytases have high binding affinities for substrates that contain neighboring phosphate groups such as phytic acid (Kerovuo, 1998; Kim et al., 1998; Shin et al., 2001). Purple acid phosphatases

are a type of metalloenzymes which contain a purple acid phosphatase as the active site. The GmPhy (EC 3.1.3.2), isolated from cotyledons of germinating soybeans, is the only PAP with significant phytase activity (Klabunde et al., 1996; Hegeman and Grabau, 2001). The most common phytase class is the HAP (E.C. 3.1.3.8 and 3.1.3.26). Phytases supplemented in animal feed are included in this class. These phytases act in a 2-step mechanism to hydrolyze phosphomonoesters and share a common active site (Ullah, 1991).

Histidine acid phosphatases can be further characterized by their mechanistic activity as 3-phytases (EC 3.1.3.8) or 6-phytases (EC 3.1.3.26). The 6-phytases initiate hydrolysis on the phosphomonoester bond at the $_{L}$-6 ($_{D}$-4) position of phytic acid **(Figure 2.17)**. Whereas, 3-phytases begin at the $_{D}$-3 position **(Figure 2.18**; Brench-Pedersen et al., 2002). Following initial hydrolysis, phytases attack the penta*kis*phosphate adjacent to the free hydroxyl group, which results in *myo*-inositol-tetra-, -tris-, -bis-, and finally mono*kis*phosphate, as well as the liberation of phosphate groups **(Figure 2.16)**. For example, a 3-phytase would initially form a $_{D}$-*myo*-inositol-(1,2,4,5,6)-penta*kis*phosphate and a subsequent $_{D}$-*myo*-inositol-(1,2,5,6)-tetra*kis*phosphate (Irvin and Cosgrove, 1972).

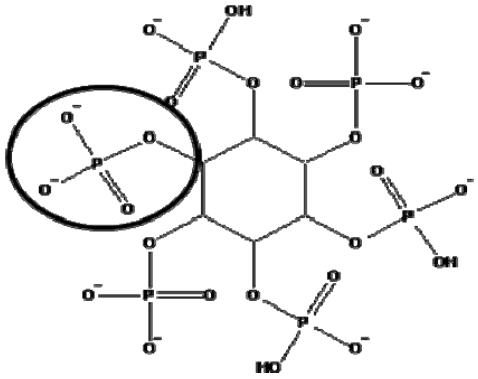

Figure 2.17. Phytate. A 6-phytase hydrolyzes the highlighted 6-phosphate group first.

adapted from Raboy, 2003

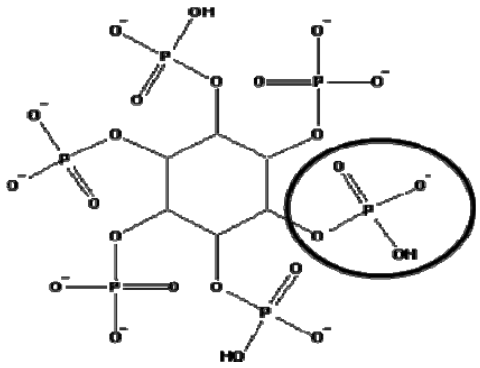

Figure 2.18. Phytate. A 3-phytase hydrolyzes the highlighted 3-phosphate group first.

adapted from Raboy, 2003

Fungal Phytases

The first generation of commercially available phytase was derived and produced from fungi such as *Aspergillus niger* and *Asperigillus ficuum.* When fungal organisms are grown under phosphate limiting conditions with corn starch as the sole carbon source, they produce 3 extracellular acid phosphatases, which can be produced via promoter replacement replication (Ullah and Gibson, 1987; van Hartingsveldt et al., 1993). These catalytically effective enzymes are 3-phytases. Fungal phytases were shown to improve total or phytate phosphorus digestibility up to 40% in pigs of various ages supplemented with 500 phytase units (FTU)/kg phytase on a corn-soybean meal based diet (Harper et al., 1997; Kemme et al., 1997; Zhang et al., 2000). In addition, corn-soybean meal poultry diets supplemented with 500 FTU/kg of a fungal phytase improved total or phytate phosphorus digestibility up to 70% (Tamim et al., 2004; Zyla et al., 2004). However, the pH profiles of these phytases do not match the pH of the gastrointestinal system. The pH profile for fungal phytases contains a peak at 5.5 and a valley at 3.5 (Ullah and Gibson, 1987), which is approximately the pH of the monogastric stomach (Yi and Kornegay, 1996). This required fungal phytases to be fed

at high inclusion levels (Lei and Stahl, 2001). In addition, fungal phytases are not thermo-stable and may be unstable during heat processing like pelleting (Ullah and Mullaney, 1996). Therefore, new phytases were developed from bacterial sources such as *Escherichia coli.*

Bacterial Phytases

Bacterial-derived phytases are better suited for the animal feed industry than fungal phytases. These 6-phytases contain a higher specific activity for phytic acid and are more thermo-stable than fungal phytases. In addition, the pH profiles for *Escherichia coli*-derived phytases contain a peak at 3.5, which is closer to the pH of the monogastric stomach (Griener et al., 1993). These 6-phytases are derived from *Escherichia coli* and are cloned and inserted into yeast such as *Saccharomyces cerevisiae, Streptomyces lividans* or *Pichia pastoris* for commercial production **(Figure 2.19**; Rodriguez et al., 1999; Stahl et al., 2003). Furthermore, inclusion of *E. coli*-derived phytases at 150 to 450 FTU/kg in wheat-soybean meal diets improved total or phytate phosphorus digestibility in poultry to 40% (Silversides et al., 2004). At an inclusion rate of 500 FTU/kg, phosphorus digestibility in broilers increased to 70% (Onyango et al., 2005). In addition, young pigs fed *E. coli*-derived phytase at low inclusion levels had an improved phosphorus digestibility of 50% (Adeola et al., 2004; Veum et al., 2006).

Figure 2.19. *E.coli*-derived phytase production in a yeast expression species.

Ethanol Production

There are 2 main systems to produce ethanol from cereal grains: wet milling and dry grind. Dry grind plants result in 2 major products: ethanol and distillers dried grains with solubles (DDGS). Dry grind ethanol production begins with the grinding of cereal grains and the subsequent addition of water and enzymes such as amylase. This begins the digestion of the starch, which comprises over 60% of the corn kernel, into sugars such as maltose, maltotriose, glucose, and limit dextrins (Sreenath, 2006). After a cooking and cooling process, yeast is added in order to ferment the sugars. This is followed by a distillation process to recover ethanol. The solid fraction is dried to become DDGS **(Figure 2.20;** Belyea et al., 2004). In fact, every bushel of corn (25.4 kg) converted to ethanol produces 8.2 kg of DDGS (Baker and Zahniser, 2006). As ethanol production increases, so does the production of by-products. By 2011 it is projected that DDGS production will reach over 40 million metric tons (Westcott, 2007). In order for ethanol production to be sustainable, the industry must find viable uses for the DDGS it produces.

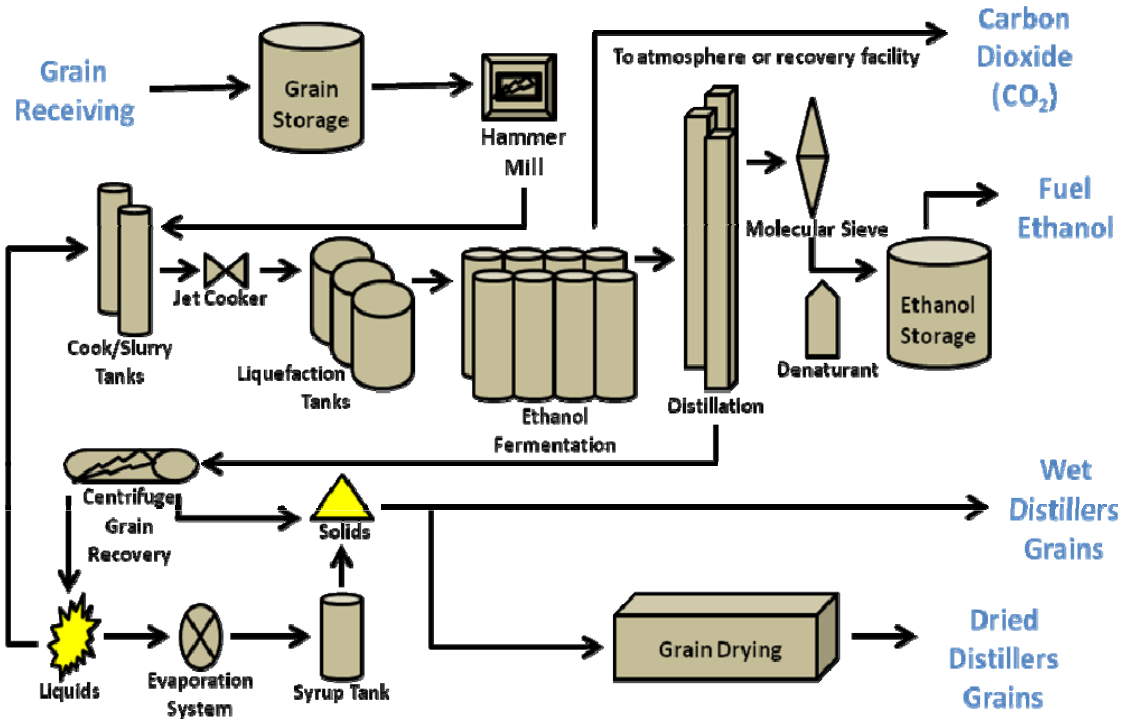

Figure 2.20. Dry mill ethanol production.

adapted from Castle Rock Renewable Fuels, 2008

Distillers Dried Grains with Solubles

As the cost of fossil fuel continues to rise, there is a growing demand for the production of renewable sources of fuel such as ethanol. It is projected that in 2010 there will be over 7 billion tons of ethanol produced in the U.S. Currently in the U.S., ethanol is mainly produced from corn. One bushel of corn (25.4 kg) can be converted into 10.6 L (2.8 gallons) of 90% ethanol (Yoder and Tolman, 2003; Mosier et al., 2005; Baker and Zahniser, 2006). In order to meet the projected needs of ethanol production, the U.S. must process 2.6 billion bushels of corn per year. This is 1.2 billion more bushels per year of corn used in the ethanol industry than in 2005 (Yoder and Tolman, 2003; Baker and Zahniser, 2006). In

addition to the increased need for corn in ethanol production, the constant need for corn in the animal feed industry has resulted in a price increase **(Figure 2.21)**. Corn price went from $2.00 per bushel in 2005 to over $7.00 per bushel in June 2008 (Leibtag, 2008). The price increase has stimulated a search for alternative energy sources for animal feed.

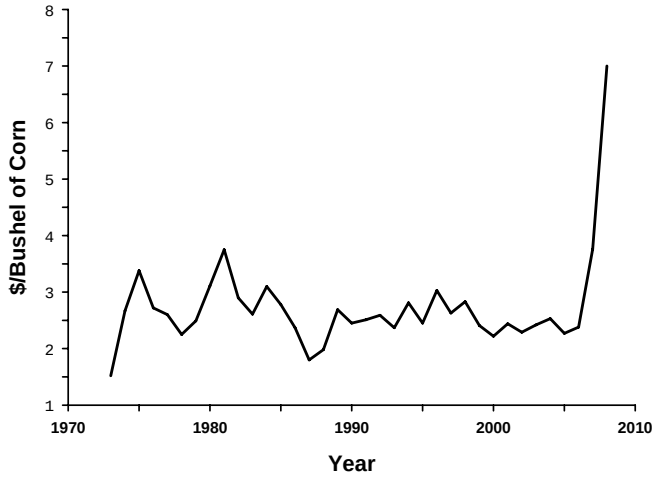

Figure 2.21. Corn price from 1970 projected to 2010.

adapted from Yoder and Tolman, 2003

Composition of Distillers Dried Grains with Solubles as an Ingredient in Monogastric Diets

When starch is removed from corn during ethanol processing, the remaining nutrients are concentrated nearly 3-fold. For example, crude fat in DDGS is 27.7% compared to 8.3% in corn (NRC, 1998; Spiehs et al., 2002). This results in a product high in protein and NSP content, and low in water soluble polysaccharides (WSP) and phytate. As a consequence, the inclusion rate for DDGS is lower in monogastric diets. Compared to corn, the low content of WSP makes DDGS a lower energy source for monogastrics. However, this composition enables DDGS to be a suitable feed source for ruminant animals. As the production of

DDGS increases, the available ruminant feed market has become saturated (NCGA, 2008). The monogastric feed market may be a possible outlet to dispose of this excess DDGS not being used in ruminant feed.

Starch removal during ethanol processing also has an effect on DDGS protein content. Distillers dried grains with solubles contains 30% CP; compared to 8.5% for corn (NRC, 1998; Spiehs et al., 2002). However, the lysine content in DDGS is variable due to the extremely high temperatures (315^{o}C) and exposure time used to dry DDGS. Excessive thermo-trauma results in diminished amino acid digestibility (McGinnis and Evans, 1947; Warnick and Anderson, 1968). This is a result of the Maillard reaction. As the DDGS is dried, lysine and reducing sugars undergo a series of chemical reactions in which the nucleophilic group of the amino acid reacts with the carbonyl group in the reducing sugar **(Figure 2.22)**. The resulting product shows the physical characteristic of browning (Bedinghaus and Ockman, 2006; Murata et al., 2008). However, when lysine digestibility was tested in chicks, the results showed an availability of 80% in DDGS which is comparable to 81% in corn (Lumpkins and Batal, 2005). This indicates that a careful DDGS drying process does not affect lysine availability in the product. Furthermore, DDGS contains increasing levels of nonessential amino acids. The excess nonessential amino acids in DDGS cause the animal to be in a positive nitrogen balance. In order to rebalance nitrogen in the animal, the excess nitrogen is excreted; inevitably, increasing the amount of nitrogen in the manure (both urine and feces; Whitney and Shurson, 2004). Due to CWA regulations on nitrogen content in manure, a practical inclusion rate of 20% is suggested for swine (Shurson et al., 2004). This allows DDGS to be a partial substitute for corn and soybean meal in monogastric diets.

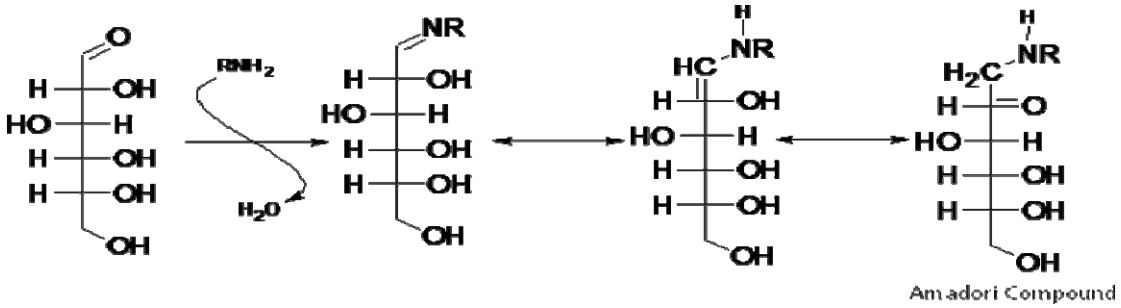

Figure 2.21. Initial step in the Maillard reaction. RNH_2 represents an amino acid with R depicting an amino acid side group.

adapted from Cardelle-Cobas et al., 2005

Although DDGS could be used as a partial protein source for monogastrics, without enzymatic help it would be a poor energy source. By removing starch during the ethanol process, the carbohydrate energy source is removed; leaving only protein and fat as possible energy sources. The result is a decreased ME in DDGS for monogastrics compared to corn. The ME of DDGS is 2,820 kcal/kg for swine compared to 3,420 kcal/kg for corn (NRC, 1998). For poultry the ME is 2,480 kcal/kg for DDGS and 3,350 kcal/kg for corn (NRC, 1994). The lowered ME is a consequence of increased crude fiber which is nearly 9% due to a high NSP content (Spiehs et al., 2002). Monogastrics do not produce NSPases and microbial fermentation does not occur prior to the small intestine. This results in a decreased capacity to utilize NSP as an energy source. Dietary inclusion of non-starch polysaccharidases may improve the energy content of DDGS by cleaving the β-linkages of NSP to release reducing sugars which can be absorbed by monogastrics.

In the corn kernel, the majority of phytate phosphorus is located in the starchy endosperm or the germ (O'Dell et al, 1972). During ethanol processing, starches are

hydrolyzed, which may release some of the phytate phosphorus. The DDGS product, results in higher non-phytate phosphorus content than corn. In fact, bioavailability of phosphorus in DDGS is 77% for swine and 72% for poultry compared to 14% in corn for swine and poultry (NRC, 1994, 1998). However, recent studies in pigs and poultry have shown increased phosphorus bioavailability compared to the values reported by the NRC (1994, 1998). Values for phosphorus bioavailability have been reported as high as 82% for DDGS in poultry (Martinez Amezcua et al., 2004) and 90% for swine (Shurson et al., 2004). Studies in wheat and barley have shown that fermentation time and temperature during soaking may have a direct effect on natural phytase activity (Carlson and Poulsen, 2003). *Saccharomyces cerevisiae* is a yeast species used in the production of ethanol (Hector et al., 2007; and Belyea, 2004). This species of yeast also produces a phytase enzyme (Brenda Enzyme Database, 2008). Thus, explaining the increased phosphorus bioavailability in DDGS compared to corn.

Currently, DDGS is being recommended at a maximum inclusion level of 15% for poultry and 20% for swine. At levels higher than 15%, there was an overall 3% reduction in egg production, and consistent decrease in egg production after wk 32 of production in poultry (Roberson, 2003; Lumpkins et al., 2005; Roberson et al., 2005). Feeding chicks a corn-soybean meal diet with 18% DDGS resulted in decreased weight gain and gain:feed ratio at 16 d and decreased weight gain at 42 d compared to chicks fed a similar diet with DDGS included at 0 to 12% (Lumpkins et al., 2004). Grow-finish pigs fed corn-soybean meal based diets with 20 to 30% DDGS had a reduced average daily gain compared to pigs fed the same diet with 0 to 10% DDGS (Shurson et al., 2004). Inclusion levels above 20% of the diet increase concerns of soft pork fat due to the high unsaturated oil content in DDGS

(Shurson et al., 2004). However, in many of the studies the ingredients were not kept balanced. In order for DDGS to increase in the diet, another ingredient must be removed. In diets where DDGS took the place of corn, the energy level decreased as the amount of DDGS increased. Likewise, in diets where DDGS displaced soybean meal, the quality of amino acids decreased as DDGS increased, especially lysine (Roberson, 2003; Whitney and Shurson, 2004). This may be a possible explanation for the decreased performance results.

Conclusion

In conclusion, the addition of phytase to monogastric diets allows for an increase in the available phosphorus. Inclusion of DDGS could be increased in monogastric diets with the addition of NSPases without a decrease in ME of the diet. The combination of phytase and NSPases in monogastric diets including DDGS could allow for an increase in both available phosphorus and ME.

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Chapter III

Effect of Phytase on Non-Starch Polysaccharidase Activities in Distillers Dried Grains with Solubles

Abstract

As environmental concerns increase in the U.S., there is a push to go "green". This has fueled the development of ethanol production in the U.S. as a renewable source of energy. In the U.S., corn is used to produce ethanol. Therefore, displacement of corn used in the animal industry to ethanol production has, inevitably caused an increase in the price of corn. Replacing conventional ingredients, such as corn, in monogastric diets with lower cost by-products can be attractive economically. The objective of this study was to determine if exogenous enzymes that free reducing sugars from fiber will increase the dispensability of bound nutrients in the economically favorable by-product of ethanol production, distillers dried grains with solubles (DDGS). Effects of non-starch polysaccharidase (NSPase) inclusion rates and combinations with phytase on the ability to release reducing sugars were investigated. Distillers dried grains with solubles was tested *in vitro* in combination with hemicellulase (0, 0.1, 1, and 10 U/g of DDGS), cellulase (0, 1.5, 15, and 150 U/g of DDGS), xylanase (0, 2.5, 25, and 250 U/g of DDGS), β-glucanase (0, 0.006, 0.06, and 0.6 U/g of DDGS), and phytase (0, 250, 500, and 1,000 FTU/kg of DDGS). Results indicated an inhibitory effect of phytase on the ability of NSPases to release reducing sugars from DDGS ($P = 0.0004$). A second study was conducted to determine individual enzyme kinetics of NSPase on pure substrates when combined with increasing concentrations of phytase. Cellulase ($P = 0.03$), xylanase ($P = 0.09$), and β-glucanase ($P =$

0.06) combined with increasing concentrations of phytase showed a reduction in the velocity of reducing sugars release from pure substrate. However, we speculate that due to the inability to reach V_{max}, the Lineweaver-Burk results were inconclusive. The $-1/K_m$ was calculated for all enzymes, and the presence of phytase increased $-1/K_m$ for xylanase ($P = 0.006$). However, $-1/K_m$ did not change for cellulase ($P = 0.62$), β-glucanase ($P = 0.20$), and hemicellulase ($P = 0.13$). In addition, the $1/V_{max}$ was calculated for all enzymes, and the presence of phytase decreased $1/V_{max}$ for cellulase ($P = 0.03$) and β-glucanase ($P = 0.01$). However, the presence of phytase resulted in no change for the $1/V_{max}$ for xylanase ($P = 0.81$) and hemicellulase ($P = 0.14$). The slopes of the regression lines for the Lineweaver-Burk plots showed no effect of the presence of phytase for cellulase ($P = 0.40$) and hemicellulase ($P = 0.27$). However, the presence of phytase decreased the linear slope for xylanase ($P = 0.006$), and increased the linear slope for β-glucanase ($P = 0.006$). In summary, phytase appears to act as an inhibitor of NSPase activity in the *in vitro* digestion on DDGS; however, inconsistent results from the inhibition curves preclude us from determining the type of inhibition.

Introduction

The production of ethanol as a fuel source from corn removes the starch fraction in the grain and leaves distillers dried grains with solubles (DDGS) as a by-product (Belyea et al., 2004). Distillers dried grains with solubles is higher in non-starch polysaccharides (NSP), crude protein (CP), crude fat (CF), and minerals than in the parent grain (Batal and Dale, 2003; Batal and Dale, 2006; Stein et al., 2006). Monogastrics do not digest feedstuffs high in NSP efficiently (Barrera et al., 2004). As a result, the metabolizable energy (ME) of DDGS is lower than in corn

(NRC, 1998). The recent amendments to the Clean Water Act have put limitations on the amount of phosphorus that can be applied to land from manure. Monogastrics do not produce endogenous phytase (Golovan et al., 2001). Therefore, they are inefficient in hydrolyzing phytate (Pointillart et al., 1984), which results in a low phosphorus digestibility of grains (NRC, 1998). The addition of exogenous phytase to monogastric diets increases the bioavailability of phosphorus in grains and allows for lower inorganic phosphorus supply to the diet which translates to lower amount of phosphorus excreted in feces (Augspurger et al., 2003).

There is an economic drive to replace conventional ingredients in monogastric diets with low-cost by-products such as DDGS. However, the low nutrient digestibility caused by the increased presence of NSP and phytate in these by-products could be resolved through supplementation of non-starch polysaccharidase (NSPase) and phytase to the monogastric diet. The current study tested the hypothesis that the nutrient availability of DDGS can be improved with NSPase and phytase.

Materials and Methods

NSPase Activity and pH

Hemicellulase, cellulase, xylanase, and β-glucanase were donated by Bio-Cat Inc. (Troy, VA). Enzyme activity was determined at 39°C over a pH range of 2.0 to 8.0, in 0.5 pH unit increments. A 0.1 M sodium citrate-phosphate buffer was prepared for use in the determination of NSPase activity as follows: citrate phosphate buffer (Sigma-Aldrich Chemical Co., St. Louis, MO) was dissolved in distilled water, and the pH was adjusted with sodium hydroxide. The

determination of enzyme activity was performed in triplicate using purified substrates dissolved or suspended in distilled water as previously described (Colombatto et al., 2003). Briefly, the substrates were 1% (wt/vol) carboxymethylcellulose (sodium salt, low viscosity, CAS 9004-32-4, EMD Biosciences Inc., Madison, WI) for hemicellulase, 2% (wt/vol) carboxymethylcellulose (EMD Biosciences) for cellulase, 2% (wt/vol) oat spelts (CAS 9014-63-5, Sigma-Aldrich) for xylanase, and 1% (wt/vol) β-glucan from barley (medium viscosity, CAS 9041-22-9, Sigma-Aldrich) for β-glucanase. One gram of powdered hemicellulase, cellulase, xylanase, or β-glucanase was extracted with 25 mL of distilled water at 4°C under constant stirring for 5 min. Enzyme extractions were kept in ice for up to 12 h while in use. Fresh enzyme extracts were prepared before each assay. An aliquot of diluted enzyme (50 μL) and substrate or standard (50 μL) were incubated with 0.1 M sodium citrate-phosphate buffer (100 μL) at each pH for 15 min at 39°C using a water bath (Isotemp 210, Thermo-Fisher Scientific Inc., Pittsburgh, PA). Afterwards, reducing sugars were quantified using the method described below. One unit of activity was defined as the amount of enzyme required to release 1 μmol equivalent xylose or glucose per minute per gram of enzyme product, under the conditions of the assay.

NSPase Activity and DDGS

The ability of each NSPase to release reducing sugars from DDGS as the substrate was evaluated at different concentrations. The DDGS product Dakota Gold BPX was donated by Poet Nutrition (Sioux Falls, SD). All reactions were performed in triplicate at 39°C and pH 6.8, to mimic the conditions of the porcine small intestine. Concentration of enzymes ranged from 0.001 to 120 U/g for hemicellulase, 0.006 to 1.2 U/g for β-glucanase, and 0.07 to 633 U/g for

cellulase and xylanase. An aliquot of diluted enzyme (50 μL) and DDGS (0.025 g) were incubated with 0.1 M sodium citrate-phosphate buffer pH 6.8 (100 μL) for 15 min at 39°C using a water bath (Thermo-Fisher). Afterwards, reducing sugars were determined using the method described below. These results were used to determine the activity of NSPase to be used in the *in vitro* digestion assay.

Phytase Activity

The phytase product, Optiphos, was donated by JBS United, Inc. (Sheridan, IN). The activity of Optiphos was determined at 39°C at pH 6.8, to mimic the conditions of the porcine small intestine. A 0.2 M citrate buffer was prepared for use in the determination of phytase activity as follows: sodium citrate (CAS 6132-04-3, Sigma-Aldrich) was dissolved in distilled water and the pH was adjusted to 6.8 with 0.2 M citric acid monohydrate (CAS 5949-29-1, Sigma-Aldrich). The determination of enzyme activity was performed in triplicate using 5.1 mM sodium-phytate (CAS 14306-25-3, Pfaltz & Bauer, Inc., Waterbury, CT) dissolved in 0.2 M citrate buffer pH 6.8 as substrate (Augspurger et al., 2003). Briefly, powdered phytase (0.4 g) was extracted in 10 mL of 0.2 M citrate buffer at 4°C under constant stirring for 1 h. Afterwards, the mixture was centrifuged in an Eppendorf centrifuge (5810R, Eppendorf, Westbury, NY) at 3,000×g for 15 min at 4°C. Enzyme extract was kept in ice for up to 12 h, while in use. Fresh phytase extract was prepared before each assay. Aliquots of diluted phytase (500 μL) and substrate (500 μL) were incubated for 15 min at 39°C using a water bath (Thermo-Fisher). Next, 1 mL of 15% (wt/vol) trichloroacetic acid (TCA; CAS 76-03-9, Acros Organics, Geel, Belgium) in distilled water was added to each tube to stop the reaction. Then, tubes were vortexed and

centrifuged in an Eppendorf centrifuge 5810R at 3,000×g for 5 min at ambient temperature. Afterwards, the concentration of inorganic phosphorus was quantified using the method described below. One phytase unit (FTU) was defined as the amount of phytase required to release 1 μmol of inorganic phosphorus per minute from sodium-phytate at 39°C.

Phytase Activity and DDGS

The ability of phytase to release inorganic phosphorus from DDGS as the substrate was evaluated at different phytase concentrations. All reactions were performed in triplicate at 39°C and pH 6.8, to mimic the conditions of the porcine small intestine. Concentration of phytase ranged from 100 to 1,000 FTU/kg. An aliquot of diluted phytase (500 μL) and DDGS (0.025 g) was incubated for 15 min at 39°C using a water bath (Thermo-Fisher). Next, 1 mL of 15% (wt/vol) TCA in distilled water was added to each tube to stop the reaction. Then, tubes were vortexed, and centrifuged in an Eppendorf centrifuge 5810R at 3,000×g for 5 min at ambient temperature. Supernatants were collected for colorimetric determination of inorganic phosphorus concentration using the method described below. These results were used to determine the activity of phytase to be used in the *in vitro* digestion assays.

Swine In Vitro *Digestion*

The individual and combined ability of hemicellulase (0, 0.1, 1, and 10 U/g of DDGS), cellulase (0, 1.5, 15, and 150 U/g of DDGS), xylanase (0, 2.5, 25, and 250 U/g of DDGS), and β-glucanase (0, 0.006, 0.06, and 0.6 U/g of DDGS) to release reducing sugars from DDGS was

evaluated in the presence of phytase (0, 250, 500, and 1,000 FTU/kg of DDGS). To accomplish this objective, an *in vitro* digestion procedure that mimics the gastric and small intestinal phases of porcine digestion was used (Boisen and Fernandez 1997; Noblet and Jaguelin-Peyraud, 2007; Wilfart et al., 2007). Two phosphate buffers were prepared for use during the *in vitro* digestion. A 0.1 M phosphate buffer was used during the gastric phase and prepared as follows: monobasic (K_2HPO_4; CAS 7778-77-0, VWR International, LLC., West Chester, PA) and dibasic (KH_2PO_4; CAS 7758-11-4, VWR) potassium phosphate were separately dissolved in distilled water to achieve 0.1 M. Then, the pH of the 0.1 M K_2HPO_4 was adjusted to 6.0 with the 0.1 M KH_2PO_4. The same procedure was followed to prepare the 0.2 M phosphate buffer except the final pH was 6.8. The gastric phase of the *in vitro* digestion is briefly described: 0.1 g of DDGS and 5 mL of 0.1 M phosphate buffer pH 6.0 were combined in a screw-cap 16×125 mm borosilicate tube (Corning Inc., Corning, NY). Tubes were capped, rocked for 1 to 2 min at ambient temperature, and placed and maintained in ice during the addition of phytase and NSPase extracts. Enzyme extracts were freshly prepared as described above. Aliquots of phytase, NSPase, and distilled water (combined to a total 2,500 μL) were added to each tube. The gastric phase was simulated as follows: 6 M HCl was added to each tube to reduce pH between 2.0 and 3.0 using colorpHast pH-paper (EMD Chemicals Inc., Gibbstown, NJ). Then, 200 μL of 25 mg/mL of porcine pepsin (2000 FIP U/g, CAS 9001-75-6, Sigma-Aldrich) in distilled water and 100 μL of 5 mg/mL chloramphenicol (CAS 56-75-7, Sigma-Aldrich) in ethanol were added. Tubes were capped, vortexed, and immersed in a water bath (Thermo-Fisher) set at 42°C for exactly 2 min to bring the temperature of the slurry to 39°C. Tubes were transferred to a rocker (Boekel Rocker II, Boekel Scientific, Feasterville, PA) placed inside a dry-heat incubator set at 39°C for 1 h. The small intestinal phase was simulated as follows: upon completion of the gastric phase, 2 mL of

0.2 M phosphate buffer pH 6.8 and 1 mL of 0.6 M NaOH (CAS 1310-73-2, Thermo-Fisher) were added to adjust pH to 6.8. The pH of each tube was verified using colorpHast pH-paper (EMD). Then, 200 μL of 100 mg/mL porcine pancreatin (CAS 8049-47-6, Sigma-Aldrich) in distilled water were added, tubes were capped, vortexed, and immediately transferred to a rocker (Boekel) placed inside a dry-heat incubator set at 39°C for 4 h. After the small intestinal incubation, 200 μL of 10 mg/mL of phenylmethanesulfonyl fluoride (CAS 329-98-6, Sigma-Aldrich) in methanol were added to each tube to stop enzymatic activity. Tubes were immediately vortexed and centrifuged in an Eppendorf 5810R centrifuge at 3,000×g for 15 min at ambient temperature. Supernatants were collected and stored at -20°C until analyzed for reducing sugars using the microplate method described below.

Inhibition Curves

The ability of each NSPase to release reducing sugars from purified substrates in the presence of phytase was evaluated. All reactions were performed at 39°C and pH 6.8, to mimic the conditions of the porcine small intestine. Concentration of substrates ranged from 0.01 to 1% (wt/vol) carboxymethylcellulose for hemicellulase, 0.1 to 4% (wt/vol) carboxymethylcellulose for cellulase, 0.1 to 4% (wt/vol) xylan from oat spelts for xylanase, and 0.01 to 2% (wt/vol) β-glucan from barley for β-glucanase. In addition, three levels of phytase were used (0, 6.6×10^{-9}, and 6.6×10^{-7} FTU/mL). Enzyme extracts were prepared as described above. Aliquots of diluted NSPase (50 μL), substrate (50 μL), and phytase (50 μL) were incubated with 0.1 M sodium citrate-phosphate buffer pH 6.8 (100 μL) for 15 min at 39°C in a water bath

(Thermo-Fisher). Afterwards, reducing sugars were determined using the microplate method described below.

Determination of Reducing Sugars

Reducing sugars were determined using the Nelson-Somogyi procedure (Nelson 1944; Somogyi, 1952). Two reagents were prepared for the determination of reducing sugars. The reagents are referred to as Reagent A and B. Nelson-Somogyi Reagent A (i.e., the alkaline copper reagent) was prepared as follows: in a 100-mL volumetric flask 2.8 g of anhydrous Na_2HPO_4 (CAS 7558-79-4, EMD) and 8 g of Rochelle salt (potassium sodium tartrate tetrahydrate, CAS 6381-59-5, Mallinckrodt Chemicals Inc., Hazelwood, MO) were dissolved in about 25 mL of distilled water. Then, 10 mL of 1 N NaOH were added; volume was adjusted to 100 mL with distilled water, and allowed to stand for 1 to 2 d before it was used. Nelson-Somogyi Reagent B (i.e., the arsenmolybdate reagent) was prepared as follows: in a 100-mL volumetric flask 5 g of ammonium molybdate$\cdot 4H_2O$ (CAS 12054-85-2, Acros) were dissolved in about 50 mL of distilled water. Then, 4.2 mL of concentrated H_2SO_4 (about 18.3 M, CAS 7664-93-9, Thermo-Fisher) were added under constant stirring. Separately, 0.6 g of $Na_2HAsO_4\cdot 7H_2O$ (CAS 10048-95-0, Alfa Aesar, Ward Hill, MA) were dissolved in about 15 mL of distilled water and added to the arsenmolybdate solution under constant mixing. Finally, volume was adjusted to 100 mL with distilled water, incubated for 24 to 48 h at 37°C, and stored in a brown glass bottle. Just prior to use, 1 volume of this reagent was mixed with 2 volumes of 1.5 N H_2SO_4.

The reaction procedure began with the addition of 1 mL of Reagent A to the reaction tube (12x75 mm, Thermo-Fisher) used to determine NSPase activity with purified substrates or DDGS, boiled for 15 min, and cooled in ice for 5 min. Then, 1 mL of reagent B and 3 mL of distilled water were added. Tubes were capped, vortexed, and centrifuged in an Eppendorf centrifuge 5810R at 3,000×g for 5 min at ambient temperature. The absorbency of the resulting blue color supernatant was quantified at 770 nm using a 1-cm quartz cuvette (Thermo-Fisher) in a spectrophotometer (UV-1700 PharmaSpec, Shimadzu Corporation, Kyoto, Japan). For microplate determination of reducing sugars, an aliquot of supernatant obtained from *in vitro* digestion (50 μL) was mixed with 1 mL of reagent A in a 12×75 mm test tube (Thermo-Fisher), boiled for 15 min and cooled in ice for 5 min. An aliquot of this mixture (63 μL) was transferred into a 96-well plate (Thermo-Fisher), 60 μL of reagent B and 180 μL of distilled water were added, and well contents were mixed with a multi-channel pipette. The absorbency of the resulting blue color was quantified at 770 nm in a microplate reader (FLUOstar OPTIMA, BMG Labtech, Offenburg, Germany). For all assays, D-(+)-glucose (CAS 50-99-7, Sigma-Aldrich) was used to construct a standard curve.

Determination of Inorganic Phosphorus

Inorganic phosphorus was quantified using AOAC colorimetric method 986.24 (AOAC, 2005). In order for inorganic phosphorus to be quantified a molybdovanadate reagent is required. Preparation of the molybdovanadate reagent was as follows: in a 50-mL volumetric flask 1 g of ammonium molybdate•$4H_2O$ (Acros) was mixed with about 15 mL of hot (about 75°C) distilled water. Separately, 0.05 g of ammonium metavanadate (CAS 7803-55-6, MP

Biomedicals, Solon, OH) was dissolved in about 10 mL of hot (about 75°C) distilled water. Then, 6.25 mL of 70% (wt/vol) perchloric acid ($HClO_4$; CAS 7601-90-3, Mallinckrodt) in water were added to the ammonium metavanadate solution. Both solutions were combined, allow to cool at ambient temperature and volume was adjusted to 50 mL with distilled water.

The procedure for the determination of inorganic phosphorus was initiated with 200 μL of supernatants or standard, 400 μL of molybdovanadate reagent, and 1,400 μL of distilled water mixed in 10×75 mm test tube (Thermo-Fisher), and incubated for 10 min at ambient temperature. The absorbency of the resulting yellow color was quantified at 400 nm using a 1-cm quartz cuvette (Thermo-Fisher) in a spectrophotometer (Shimadzu). For microplate determination, 30 μL of supernatant or standard was transferred into a 96-well plate (Thermo-Fisher) with 60 μL of molybdovanadate reagent and 210 μL of distilled water. Well contents were mixed with a multi-channel pipette, and incubated for 10 min at ambient temperature. The absorbency of the resulting yellow color was quantified at 400 nm in a microplate reader (BMG Labtech). For all assays, KH_2PO_4 (CAS 7778-77-0, Thermo-Fisher) was used to construct a standard curve.

Statistical Analysis

Single-slope broken-line plots were constructed using the NLIN procedure of SAS (SAS Inst., Inc., Cary, NC; Robbins et al, 2006) for the action of each NSPase and phytase on DDGS. The experimental design for the porcine *in vitro* digestion consisted of a randomized block design with a 4×4×4×4×4 factorial arrangement of treatments (i.e., 5 enzymes, 4 NSPase and 1 phytase, each to be tested at 4 different inclusion levels). This arrangement of treatments produces a total of 1,024 treatments. Testing that many treatments is burdensome, not considering the number of

repetitions, even for an *in vitro* assay. Thus, the FACTEX procedure of SAS was used to construct a fractional factorial design that combines statistically confounded interactions and hence "minimize" the number of treatments (Box et al., 1978; SAS/QC, 2008). In this case, the factorial design was modified from full resolution of 1,024 treatments to resolution = 4 with 64 treatments, 4_{IV}^{5-2}. A fractional factorial design with resolution = 4, allows for the estimation of all main effects free of each other and free of two-factor interactions, but some two-factor interactions are confounded with each other.

Two-way interactions between phytase and each NSPase were tested using multiple linear regressions with GLM and response surface analysis using the REG procedures of SAS. Both the quadratic and linear slopes of the Michaelis-Menten were determined with GLM. The Lineweaver-Burk plots were computed as the double reciprocal of the Michaelis-Menten. The $-1/K_m$, $1/V_{max}$, and slopes were computed using the REG procedures of SAS and tested using the GLM and LSmeans procedures of SAS.

Results

NSPase Activity and pH

The NSPase activity over pH is given in **Figures 3.1-3.4**. In all profiles, there was a peak in activity around pH 5, and comparisons were made between pH 3.0 (gastric pH) and pH 7.0 (small intestinal pH). For cellulase, the specific activity at porcine gastric pH (8,764 ± 378 U/g protein) was similar (P = 0.42) to the porcine small intestine pH (8,328 ± 378 U/g protein). Xylanase showed a 69% greater specific activity (15,995 ± 477 U/g protein) in the stomach than (P < 0.0001) the specific activity in the small intestine (11,065 ± 477 U/g protein). The specific

activity of β-glucanase in the small intestine (1,186 ± 30 U/g protein) was 80% ($P < 0.0001$) of the specific activity in the stomach (1,482 ± 30 U/g protein). Hemicellulase specific activity in the small intestine (371 ± 67 U/g protein) was 52% ($P = 0.001$) of the specific activity in the stomach (713 ± 67 U/g protein).

NSPase Activity and DDGS

A broken-line analysis was used to determine enzymes values to be used in the porcine *in vitro* digestion assays. Four pre-determine enzyme concentrations were going to be used in the *in vitro* assay as follows: 1) no enzyme (i.e., 0 U/g of DDGS) as the negative control; 2) an enzyme concentration in the linear portion of the response; 3) the enzyme concentration at the break-point; and 4) an enzyme concentration in the plateau portion of the response. The amount of reducing sugars freed from DDGS increased linearly ($P = 0.0004$) with increases in cellulase concentration until the break-point of 12.4 U/g of DDGS when the reducing sugars plateaued (**Figure 3.5**). Post rounding the break-point to 15 U/g, cellulase concentrations for the *in vitro* digestion were determined to be 0, 1.5, 15, and 150 U/g of DDGS. As xylanase concentration increased, the amount of reducing sugars freed from DDGS increased linearly ($P = 0.02$) until the break-point of 21.6 U/g of DDGS when the reducing sugars plateaued (**Figure 3.6**). The break-point was rounded to 25 U/g in order for the xylanase concentrations to be determined for the *in vitro* digestion as 0, 2.5, 25, and 250 U/g of DDGS. The amount of reducing sugars freed from DDGS increased linearly ($P = 0.006$) with increases in β-glucanase concentration until the break-point of 0.06 U/g of DDGS when the reducing sugars plateaued (**Figure 3.7**). Thus, β-glucanase concentrations for the *in vitro* digestion were determined to be 0, 0.006, 0.06, and

0.6 U/g of DDGS. As hemicellulase concentration increased, the amount of reducing sugars freed from DDGS increased linearly (P = 0.05) until the break-point of 2 U/g of DDGS when the reducing sugars plateaued (**Figure 3.8**). Post rounding the break-point to 1 U/g, hemicellulase concentrations for the *in vitro* digestion were determined to be 0, 0.1, 1, and 10 U/g of DDGS.

Phytase Activity and DDGS

The amount of inorganic phosphorus freed from DDGS did not increase (P = 0.15) with increases in phytase concentration. The calculated break-point for phytase was 0.32 FTU/kg of DDGS when the inorganic phosphorus plateaued (**Figure 3.9**). Thus, the predetermined values to use in the *in vitro* digestion would have been 0, 0.032, 0.32, and 3.2 FTU/kg of DDGS. Because these phytase values are markedly different from commercial inclusion levels, we decided to use the commercial levels of 0, 250, 500, and 1,000 FTU/kg of DDGS to increase the applicability of the results to commercial animal production. Previously reported maximum practical inclusion levels of DDGS are approximately 20% of the total diet for monogastrics (Roberson, 2003; Lumpkins et al., 2004; Shurson et al., 2004). This leaves 80% of the diet for conventional ingredients such as corn and soybean meal. Corn contains 0.20% and soybean meal contains 0.42% phytate phosphorus (NRC, 1994; 1998). For this reason, we hypothesize that inclusion levels of phytase in commercial animal production will not change from the currently recommended levels.

Swine In Vitro *Digestion*

The interaction of phytase by NSPase only showed an effect with hemicellulase (P = 0.02; **Figure 3.13B**) on the release of reducing sugars from DDGS during the *in vitro* digestion. However, the interaction of phytase and cellulase (P = 0.52; **Figure 3.10B**), phytase and xylanase (P = 0.89; **Figure 3.11B**), and phytase and β-glucanase (P = 0.24; **Figure 3.12B**) did not show an effect on the release of reducing sugars. Interestingly, replication as a main effect (P < 0.001) also showed an effect on the release of reducing sugar. This is most likely due to the methods of the protocol, which requires daily extraction of the enzymes. This could allow for variations in the enzyme activity. As a result of the effect of replication, we chose to run a hypothesis tests to determine the effect of phytase without the influence of replication. This showed that the interactions of phytase by NSPase did not have an effect on the release of reducing sugars: phytase and hemicellulase (P = 0.37), phytase and cellulase (P = 0.57), phytase and xylanase (P = 0.97), and phytase and β-glucanase (P = 0.18).

The main effects of phytase (P = 0.004) and hemicellulase (P < 0.0001; **Figure 3.13A**) showed an effect on the release of reducing sugar during the *in vitro* digestion. However, the main effects of cellulase (P = 0.39; **Figure 3.10A**), xylanase (P = 0.89; **Figure 3.11A**), and β-glucanase (P = 0.70; **Figure 3.12A**) did not. Again due to the effect of replication, we chose to run a hypothesis tests to determine the effect of phytase without the influence of replication. As a result, phytase (P = 0.96) did not have an effect on the release of reducing sugars. After the hypothesis test, hemicellulase (P = 0.04) continued to show an effect on the release of reducing sugars. Furthermore, cellulase (P = 0.49), xylanase (P = 0.95), and β-glucanase (P = 0.58) did not show an effect on the release of reducing sugars when replication is no longer a factor.

Inhibition Curves

Slope analysis was used to determine differences in the trend of the Michaelis-Menten graphs. The addition of the increasing levels of phytase to cellulase ($P < 0.03$; **Figure 3.14**), xylanase ($P < 0.09$; **Figure 3.15**), and β-glucanase ($P < 0.06$; **Figure 3.16**) resulted in a decrease in the velocity of the NSPases to release reducing sugars. However, when increasing levels of phytase were added to hemicellulase there was a change in the slope of the Michaelis-Menten curve ($P < 0.10$; **Figure 3.17**). Hemicellulase did not follow a typical Michaelis-Menten curve, and therefore, the results are deemed inconclusive.

The Lineweaver-Burk plots for NSPases were constructed from individual regression equations for each replication by calculating $1/V_o$ and 1/[S]. The resulting equations were used to extrapolate the x = 0 point to determine $-1/K_m$, and y = 0 point to determine $1/V_{max}$. Calculated $-1/K_m$ values were analyzed and the results for all NSPase are displayed in **Table 3.1**. The presence of phytase increased $-1/K_m$ for xylanase ($P = 0.006$). However, $-1/K_m$ did not change for cellulase ($P = 0.62$), β-glucanase ($P = 0.20$), and hemicellulase ($P = 0.13$). The calculated $1/V_{max}$ for all NSPase is displayed in **Table 3.2**. The presence of phytase decreased $1/V_{max}$ for cellulase ($P = 0.03$) and β-glucanase ($P = 0.01$). However, the presence of phytase resulted in no change for the $1/V_{max}$ for xylanase ($P = 0.81$) and hemicellulase ($P = 0.14$). The slopes of the regression lines for the Lineweaver-Burk plots (**Table 3.3**) showed no effect of the presence of phytase for cellulase ($P = 0.40$) and hemicellulase ($P = 0.27$). However, the presence of phytase resulted in a reduced slope for xylanase ($P = 0.006$) and increased slope for β-glucanase ($P = 0.006$).

Discussion

NSPase Activity and pH

For all the NSPase tested in this study (i.e., cellulase, xylanase, β-glucanase, and hemicellulase), the peak activity occurred near a pH 5. This is possible indicative of the origin of these enzymes, which were cloned from microorganisms such as *Bacillus subtilis, Ruminococcus albus,* and *flavefaciens* (Leatherwood, 1965; Bernier et al., 1983). These bacteria reside in the rumen of ruminant mammals. Ruminants fed in a feed-lot scenario can have a rumen pH around 5.0 (Gonzalez et al., 2008). In addition, these bacteria have the ability to adjust to their environmental pH for survival, and species like *Bacillus subtilis* also reside in the soil (Harwood et al., 2001). Furthermore, the increased activity at the gastric pH compared to the intestinal pH indicates the appropriateness of these enzymes for monogastric diets. Non-starch polysaccharidases added to monogastric diets can hydrolyze the β-linkages to release reducing sugars prior to the monogastric site of absorption at the small intestine. Based entirely on pH results, we expect more reducing sugars to be released when cellulase is fed to monogastrics than any of the other NSPase because cellulase is equally active at both gastric and small intestinal pH.

NSPase Activity and DDGS

Distillers dried grains with solubles have been reported to contain 16% cellulose, 8.2% xylan, 21.2% glucan (starch plus cellulose), and 17.5% hemicellulose on a dry-matter basis (Kim et al., 2008; Poet Nutrition, 2008). The addition of cellulase at 15 U/g of DDGS resulted in a 13% increase in the availability of reducing sugars. There was a 24% increase in reducing

sugars from xylanase at 25 U/g of DDGS. β-Glucanase resulted in a 10% increase in the availability of reducing sugars when added at 0.06 U/g of DDGS. The presence of hemicellulase at 1 U/g increased reducing sugars by 47%. Assuming 100% absorption of the freed reducing sugars and using the swine ME of anhydrous glucose as a reference (i.e., 3,586 kcal/kg; NRC, 1998), the resulting increase in ME for DDGS would be 108 kcal/kg for cellulase, 114 kcal/kg for β-glucanase, 224 kcal/kg for xylanase, and 393 kcal/ kg for hemicellulase. Thus, the expected ME for DDGS with NSPase addition would increased from 2,820 kcal/kg to 2,928 kcal/kg with cellulase, 2,934 kcal/kg with β-glucanase, 3,044 kcal/kg with xylanase, and 3,213 kcal/kg with hemicellulase.

Through increasing the ME of a diet with the addition of NSPase there is an improved growth performance in pigs (Fan et al., 2008). Furthermore, pigs fed hulless barley-soybean meal, also high in NSP, supplemented with 0.2% β-glucanase had an improved ileal GE digestibility (71.1%) compared to the diet without β-glucanase supplementation (64.9%; Li et al., 1996). Our results suggest that the addition of NSPase to high fibrous feed ingredients, such as DDGS, could be an efficient feeding practice to increase the energy content of by-products to monogastrics.

Phytase Activity and DDGS

The inorganic phosphorus content in the DDGS used in these experiments was 7.6 g/kg of DDGS. This is comparable to the NRC (1998) value of 7.7 g/kg of DDGS. Our results indicated a break-point at 0.32 FTU/kg of DDGS increasing the inorganic phosphorus by 55%. The break-point is much lower than the commercially suggested levels. However, the

commercial recommendations are for typical corn-soybean meal diets, which are higher in phytate. Furthermore, DDGS is more bioavailable in inorganic phosphorus than corn; 77% inorganic phosphorus bioavailability for DDGS compared to 14% for corn (NRC, 1998). The increased inorganic phosphorus bioavailability of DDGS may explain the observed low break-point. We chose to use commercially suggested levels instead of the obtained break-point results as the test levels for the *in vitro* experiment. This approach ensures the applicability of results from the *in vitro* digestion to commercial animal production since phytase recommendations are likely to remain unchanged. Phytase has been shown to continually increase the release of inorganic phosphorus at levels much higher than commercially acceptable levels in corn-soybean meal diets. For example, Adeola (2004) showed that an *E. coli*-derived phytase freed 863 μg of inorganic phosphorus/g of corn at 1,000 FTU/kg compared to 1,239 μg of inorganic phosphorus/g of corn at 4,000 FTU/kg. Furthermore, Veum (2006) also reported an increase in the amount of phosphorus absorbed in nursery-age pigs as the phytase concentration increased in the diet. Pigs fed a corn-soybean meal basal diet supplemented with phytase at 500 FTU/kg absorbed 2.28 g of phosphorus/d compared to 4.23 g of phosphorus/d in diets supplemented with phytase at 12,500 FTU/kg. Inclusion of 500 FTU/kg of phytase to a corn-soybean meal diet reduced fecal phytate phosphorus from 3.7 mg/g to 2.7 mg/g (Hill et al., 2008). When 15% DDGS was included in the diet at the expense of corn and soybean meal, fecal phytate content was comparable to the phytase-supplemented diet (2.7 vs 2.2 mg/g) indicating the lower amount phytate phosphorus in DDGS. When the DDGS diet was supplemented with 500 FTU/kg of phytase, fecal phytate concentration was significantly reduced to 1.1 mg/g. Collectively, results from this study indicate the ability of 500 FTU/kg of phytase to reduce fecal phytase phosphorus in about 1 mg/g. Furthermore, these results support our

decision to use commercially recommended levels of phytase in the *in vitro* digestion study because phytase is actively cleaving phytate from corn and soybean meal in diets containing 15% DDGS. Thus, we hypothesized that inclusion of DDGS and NSPases to will not affect current suggestions for phytase inclusion in monogastric diets.

Swine In Vitro *Digestions*

The results indicate that phytase has possible inhibitory effects on NSPase activity when DDGS is the substrate. However, a discrepancy was seen between the main effect of phytase ($P = 0.004$) and the effect of phytase without the influence of replication ($P = 0.96$). This would indicate that the inhibitory effect of phytase on the release of reducing sugars during the *in vitro* digestion was more an effect of replication or the interaction of phytase and replication than phytase. As phytase concentration increased from 0 to 500 FTU/kg of DDGS, regardless of NSPase concentration, there is a decrease in the amount of reducing sugars released from DDGS ($P = 0.04$). The lowest release of reducing sugar, regardless of NSPase, occurred when phytase was added at 500 FTU/kg of DDGS. When NSPases were added at their highest inclusion levels with phytase at 250 FTU/kg of DDGS, the release of reducing sugars was no different than in the absence of phytase for cellulase ($P = 0.11$), xylanase ($P = 0.15$), and hemicellulase ($P = 0.97$). This may indicate a more appropriate inclusion combination for phytase and NSPases.

In the absence of phytase, the release of reducing sugars from DDGS using cellulase, xylanase and β-glucanase was similar to the results obtained from the broken-line analysis. There was an initial increase, followed by a plateau in the release of reducing sugars. However, there was a discrepancy between the broken-line analysis and the *in vitro* digestion results for

hemicellulase, which showed a decrease (P = 0.05) at the break-point (2 U/g of DDGS). This discrepancy can be attributed to marked differences in assay conditions. The broken-line assay was performed without the addition of gastrointestinal enzymes at a constant pH 6.8; whereas, the *in vitro* procedure was developed to mimic the monogastric gastrointestinal system.

Many *in vivo* experiments conducted in the area of NSPase and phytase combination have also used varying phytase and NSPase concentrations without reporting individual effects like we have here. When poultry were fed a corn-soybean meal diet supplemented with about 1,600 FTU/kg phytase, 850 U/kg xylanase, 2,050 U/kg amylase, and 4,200 U/kg protease resulted in an ileal digestible energy (IDE) of 2,791 kcal/kg (Cowieson and Adeola, 2005). When phytase was removed from the diet, the IDE significantly increased to 2,925 kcal/kg. Similarly, apparent digestible energy content in 20-kg pigs was increased (3,554 vs 3,675 kcal/kg) in a wheat-based diet supplemented with 4,000 U/kg xylanase when phytase supplementation was reduced from 500 to 250 FTU/kg (Woyengo et al., 2008). The increase in IDE and apparent digestible energy when phytase was excluded or reduced from the diet supports our findings of a possible inhibitory effect of phytase on NSPase activity. However, a recent poultry study failed to demonstrate any increase in IDE when phytase was removed from a corn-soybean meal diet supplemented with 650 U/kg xylanase, 1,650 U/kg amylase, and 4,000 U/kg protease (Olukosi et al., 2007a). When nursery-age pigs were fed a complex grain and oilseed meal diet supplemented with 400 FTU/kg phytase, 250 U/kg xylanase, 150 U/kg glucanase, 0.001% amylase, 0.0003% protease, and 0.002% invertase the apparent ileal digestibility of NSP (10.1 vs 21.4%), gross energy (62.8 vs 71.4%), and phytate (59.2 vs 69.7%) were significantly increased and resulted in higher weight gain and gain:feed compared to control diet (Omogbenigun et al., 2004). These results suggest that the ability of NSPases to

degrade fiber in corn-soybean meal diets as well as in complex grain and oilseed meal diets was not impaired by the inclusion of phytase. Collectively, the results from experiments using blends of phytase, NSPases, and other enzymes are difficult to interpret because of the inability to separate the individual effect of each type of value-added enzyme.

Inhibition Curves

The results indicate that phytase has a potential inhibitory effect on the activities of NSPase on DDGS in the *in vitro* digestion. However, inconclusive results were obtained when NSPases activity was determined in the presence of phytase using pure substrates. The Michaelis-Menten graphs for cellulase ($P < 0.03$), xylanase ($P < 0.09$), and β-glucanase ($P < 0.06$) show differences in velocities. However, they do not exemplify typical inhibition graphs. Addition of a low level of phytase (6.6×10^{-9} FTU/mL) reduced velocity, a typical result for an inhibitory effect. However, increasing the phytase level (6.6×10^{-7} FTU/mL) had the opposite effect. A typical inhibitory Michaelis-Menten graph shows the highest velocity in the absence of an inhibitor, and the lowest velocity with the greatest concentration of inhibitior (Voet and Voet, 2004). The Lineweaver-Burk graphs and values for $-1/K_m$, $1/V_{max}$, and linear slope (K_m/V_{max}) were also inconclusive for cellulase, xylanase and hemicellulase. We hypothesize that this is a result of the inability of the NSPases to reach V_{max}. In hindsight, we speculate that if the concentration of phytase used was markedly higher an inhibition effect would have been obtained. Furthermore, with increased concentration of NSPase and/or higher concentrations of substrate, V_{max} would have been reached and more conclusive Lineweaver-Burk plots would have been obtained.

Although we are not certain of the mechanism(s) by which phytase is a possible inhibitor for NSPases, we have several hypotheses: (1) phytase itself, (2) one of its products, (3) remnants of commercial production in yeast, or (4) modification of the remnants during processing. It is not unreasonable to think that phytase can bind directly to the NSPase, or vice versa, but it is unlikely do their substrate affinities. The structure of NSPases do not resemble the structure of phytate or *myo*-inositol, and therefore, phytase should have little affinity to NSPase. The end products of phytase hydrolysis are phosphate groups and *myo*-inositol-bis*kis*phosphate (Brench-Pedersen et al., 2002; and Irvin and Cosgrove, 1972). The *myo*-inositol ring is a 6-carbon ring (Loewus and Murthy, 2000). Glucose is the end product of many NSPase hydrolyses, which is also a 6-carbon ring (Macfarlane and Englyst, 1986). Therefore, it may be plausible for *myo*-inositol-bis*kis*phosphate to mimic NSPase products, and hence affect the stoicheometry of the reaction.

A more likely mechanism for the inhibition of NSPase activity by phytase is due to remnants of commercial production in yeast or modification of these remnants. The phytase used here is an *E. coli*-derived phytase. The commercial production of phytases is the result of cloning the gene from the origin species and expressing it in yeast species such as *Saccharomyces cerevisiae, Streptomyces lividans,* or *Pichia pastoris* for mass production (Rodriguez et al., 1999; Stahl et al., 2003). These yeast species contain cell walls similar to those of plant species constructed of NSP (Kollár et al., 1997). In fact, dried yeast product contains 16.6% dietary fiber (Sarwar et al., 1985), which contains mannanoligosaccharides, glucans, glycans, and chitin (Lipke and Ovalle, 1998). Thus, NSPase can potentially attack the remnants of the yeast cell wall instead of the NSP substrates in DDGS.

Implications

The swine *in vitro* digestion has proven to be a successful and rapid assay to test the effects of exogenous enzyme combinations. Furthermore, the *in vitro* digestion can be used to screen monogastric diets prior to running an *in vivo* trial. Finally, the results suggest that phytase or its associated components can potentially act as inhibitors of NSPase activities *in vitro*.

Tables

Table 3.1. The calculated -1/K_m values for NSPase Lineweaver-Burk plots.

	Phytase[1]			SEM	P-value
	0	6.6×10-9	6.6×10-7		
Cellulase[2]	-0.200	-0.223	-0.27	0.050	0.62
Xylanase[2]	-0.187[a]	-0.133[a]	-0.274[b]	0.024	0.006
β-Glucanase[3]	-3.118	-1.086	-6.03	1.807	0.20
Hemicellulase[4]	-11.989	-6.728	-22.027	4.761	0.13

[1] FTU/mL
[2] 3.3×10^{-6} U/mL
[3] 1.32×10^{-9} U/mL
[4] 6.6×10^{-10} U/mL
[a,b] Letters differ at $P < 0.1$

Table 3.2. The calculated 1/V_{max} values for NSPase Lineweaver-Burk plots.

	Phytase[1]			SEM	P-value
	0	6.6×10-9	6.6×10-7		
Cellulase[2]	1.117[a]	0.995[b]	0.990[b]	0.025	0.03
Xylanase[2]	0.649	0.639	0.659	0.022	0.81
β-Glucanase[3]	0.701[a]	0.695[a]	0.643[b]	0.011	0.01
Hemicellulase[4]	1.022[a]	1.008[a]	0.906[b]	0.038	0.14

[1] FTU/mL
[2] 3.3×10^{-6} U/mL
[3] 1.32×10^{-9} U/mL
[4] 6.6×10^{-10} U/mL
[a,b] Letters differ at $P < 0.1$

Table 3.3. The linear slopes for NSPase Lineweaver-Burk plots.

	Phytase[1]			SEM	P-value
	0	6.6×10-9	6.6×10-7		
Cellulase[2]	5.295	5.003	4.464	0.397	0.40
Xylanase[2]	4.166[a]	5.066[a]	2.887[b]	0.368	0.006
β-Glucanase[3]	0.240[a]	0.570[b]	0.196[a]	0.062	0.006
Hemicellulase[4]	0.503	0.269	0.184	0.125	0.27

[1] FTU/mL
[2] 3.3×10^{-6} U/mL
[3] 1.32×10^{-9} U/mL
[4] 6.6×10^{-10} U/mL
[a,b] Letters differ at $P < 0.1$

Graphs

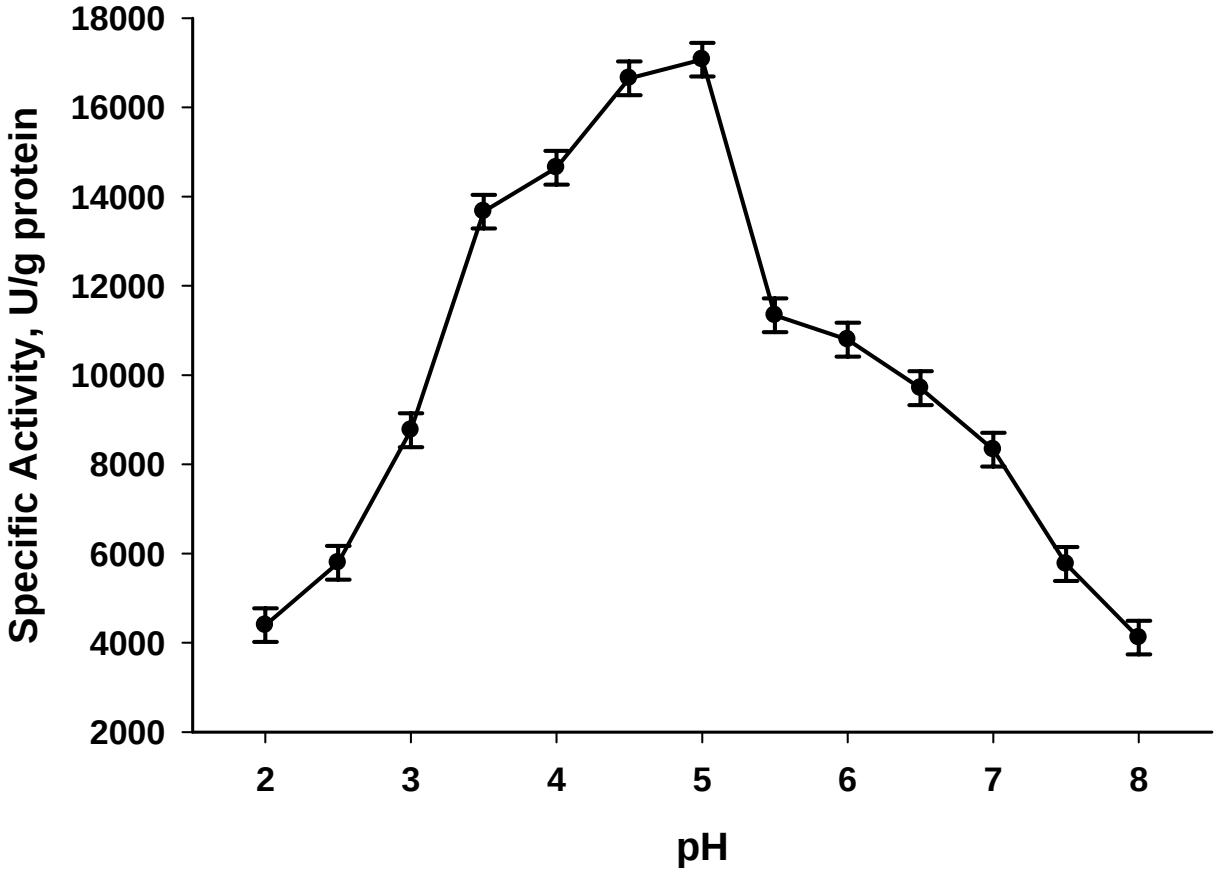

Figure 3.1. Cellulase specific activity over a pH range of 2.0 to 8.0. The peak specific activity occurred at approximately pH 5. Cellulase specific activity at porcine gastric, approximately pH 3, was similar to the specific activity at porcine small intestine, approximately pH 7 (P = 0.42).

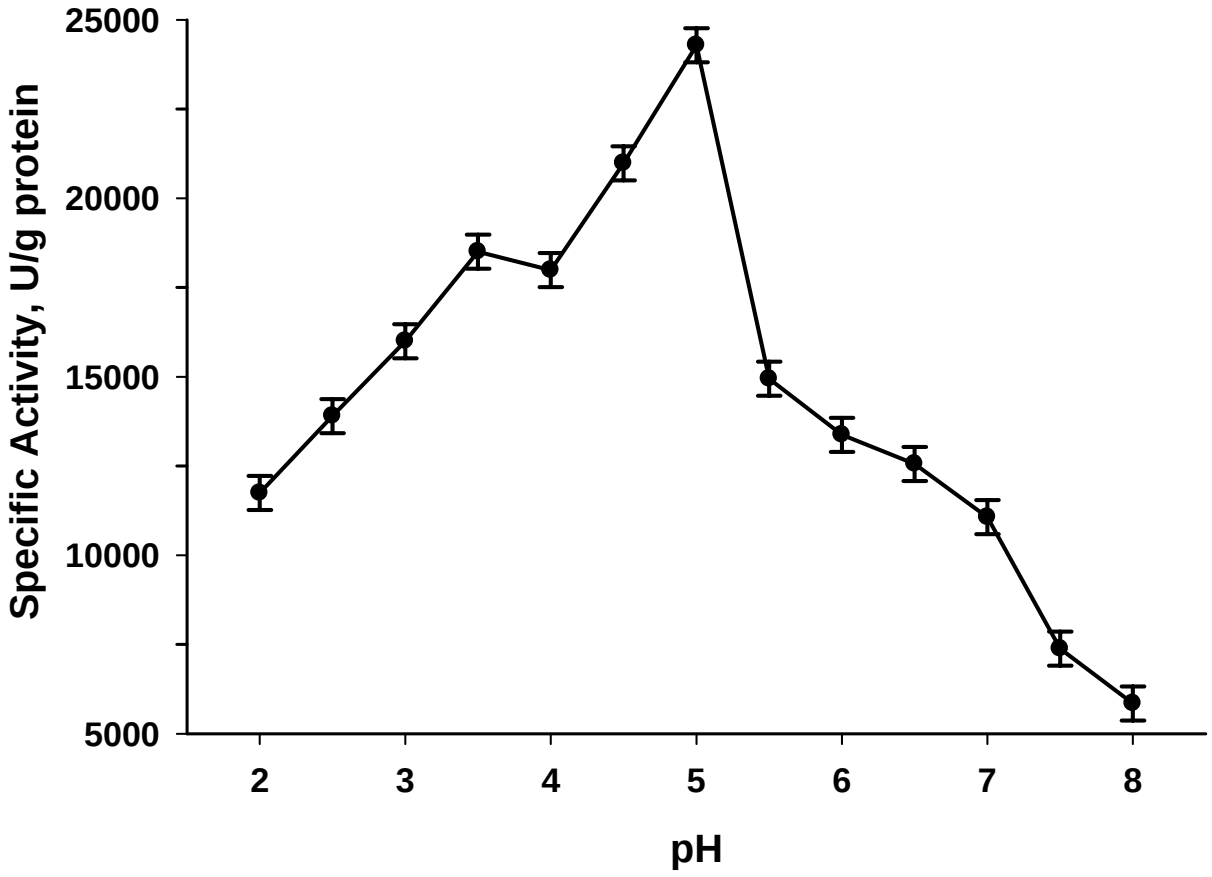

Figure 3.2. Xylanase specific activity over a pH range of 2.0 to 8.0. The peak specific activity occurred at approximately pH 5. Xylanase specific activity at porcine gastric, approximately pH 3, was greater than porcine small intestine, approximately pH 7 (P = 0.001).

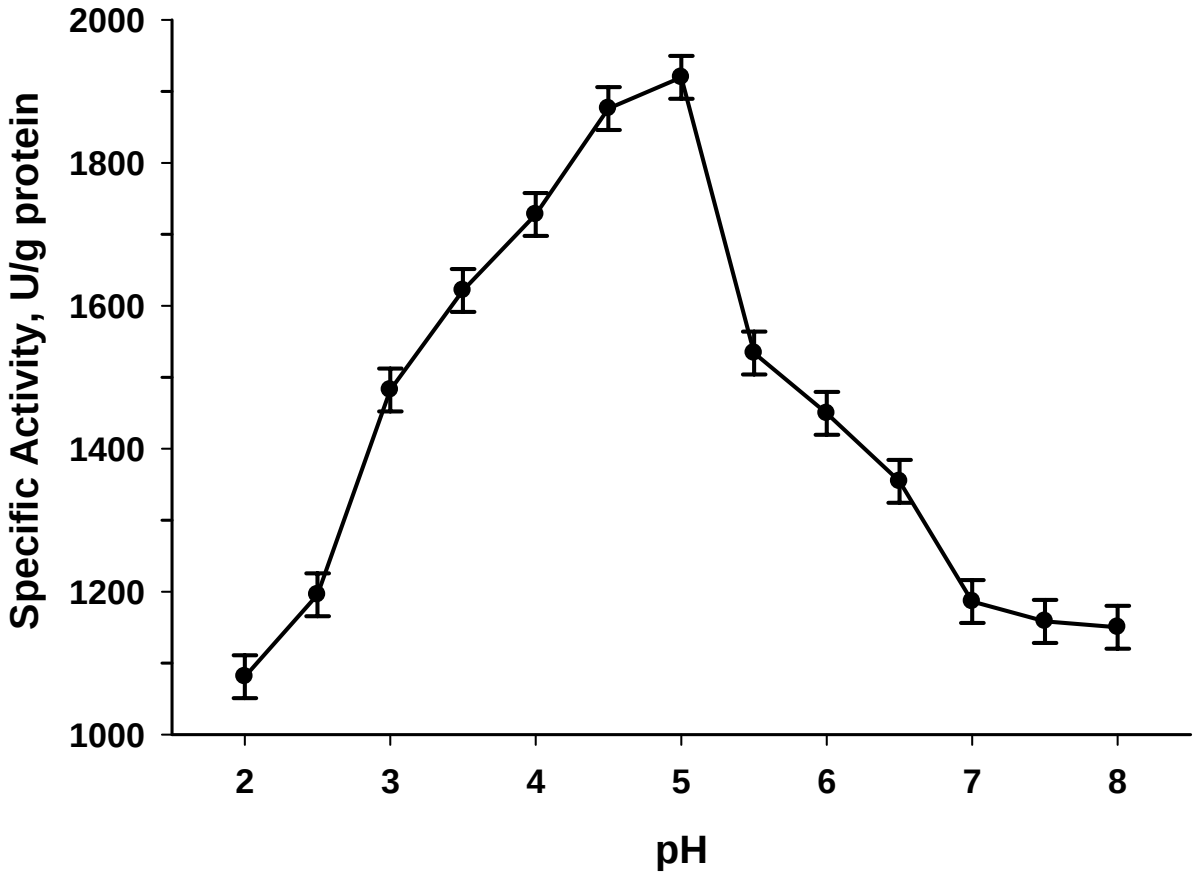

Figure 3.3. β-glucanase specific activity over a pH range of 2.0 to 8.0. The peak specific activity occurred at approximately pH 5. β-glucanase specific activity at porcine gastric, approximately pH 3, was greater than porcine small intestine, approximately pH 7 ($P < 0.0001$).

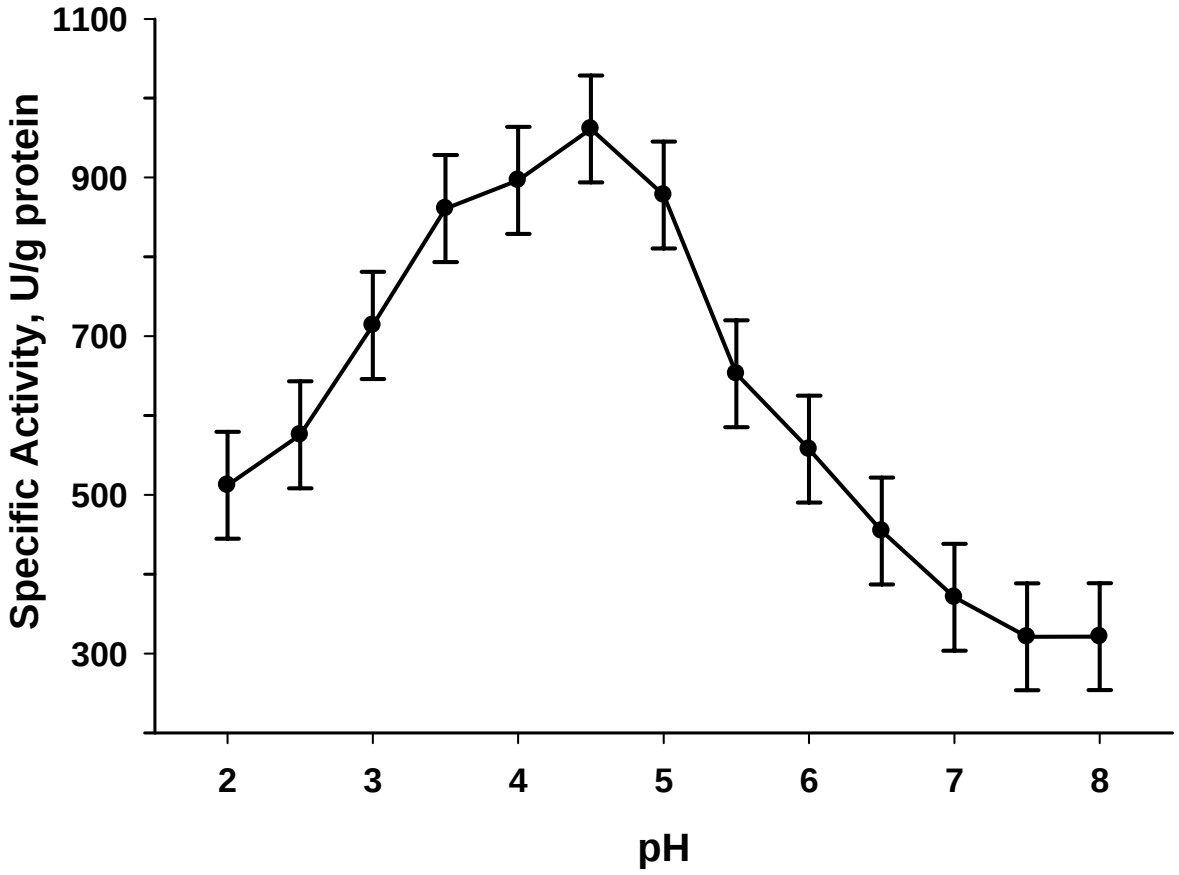

Figure 3.4. Hemicellulase specific activity over a pH range of 2.0 to 8.0. The peak specific activity occurred at approximately pH 5. Hemicellulase specific activity at porcine gastric, approximately pH 3, was greater than porcine small intestine, approximately pH 7 ($P < 0.0001$).

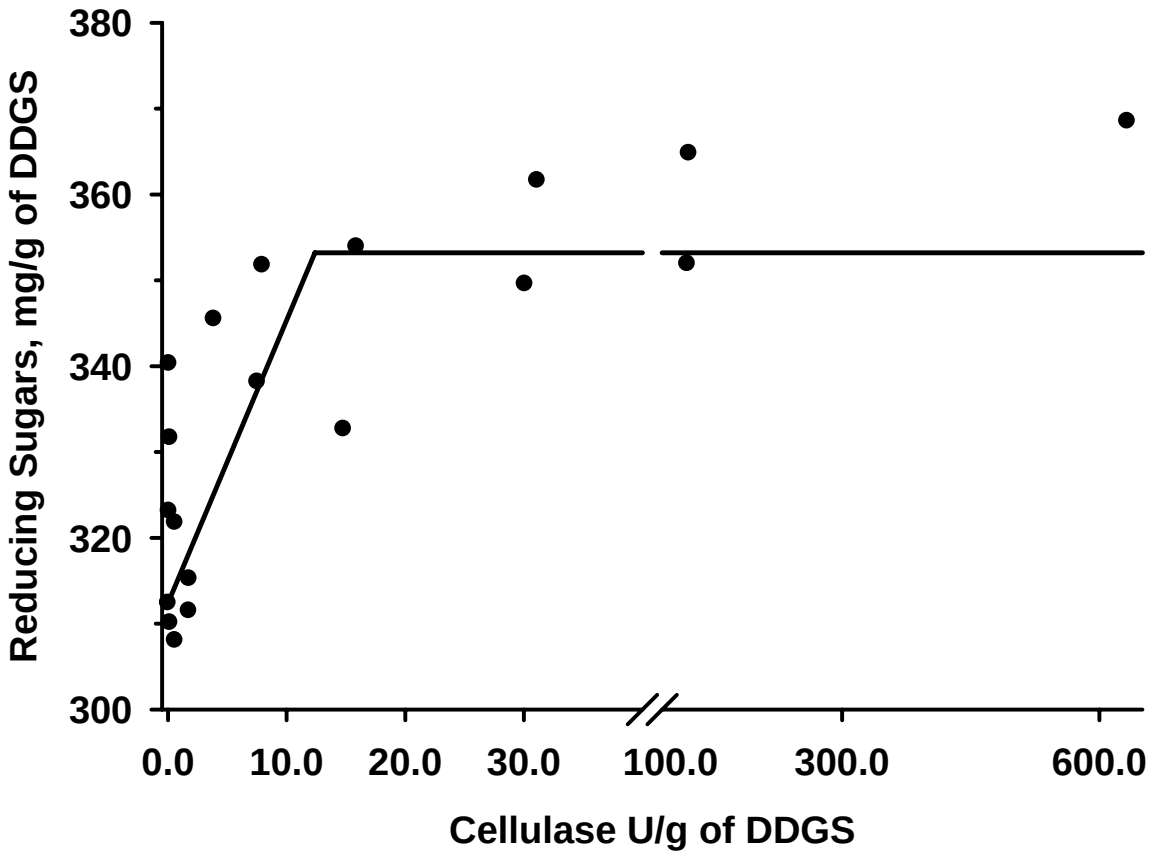

Figure 3.5. Effect of cellulase activity on the release of reducing sugars from distillers dried grains with solubles (DDGS). Increasing cellulase concentrations were incubated with DDGS at 39^{o}C for 15 min. Reducing sugars were determined using the Nelson-Somogyi procedure (Nelson 1944; Somogyi, 1952). A broken-line was fit to data to determine the break-point, which occurred at 12.39 U/g, releasing 353.2 mg/g of reducing sugars. The linear slope is 2.87, $r^2 = 0.36$.

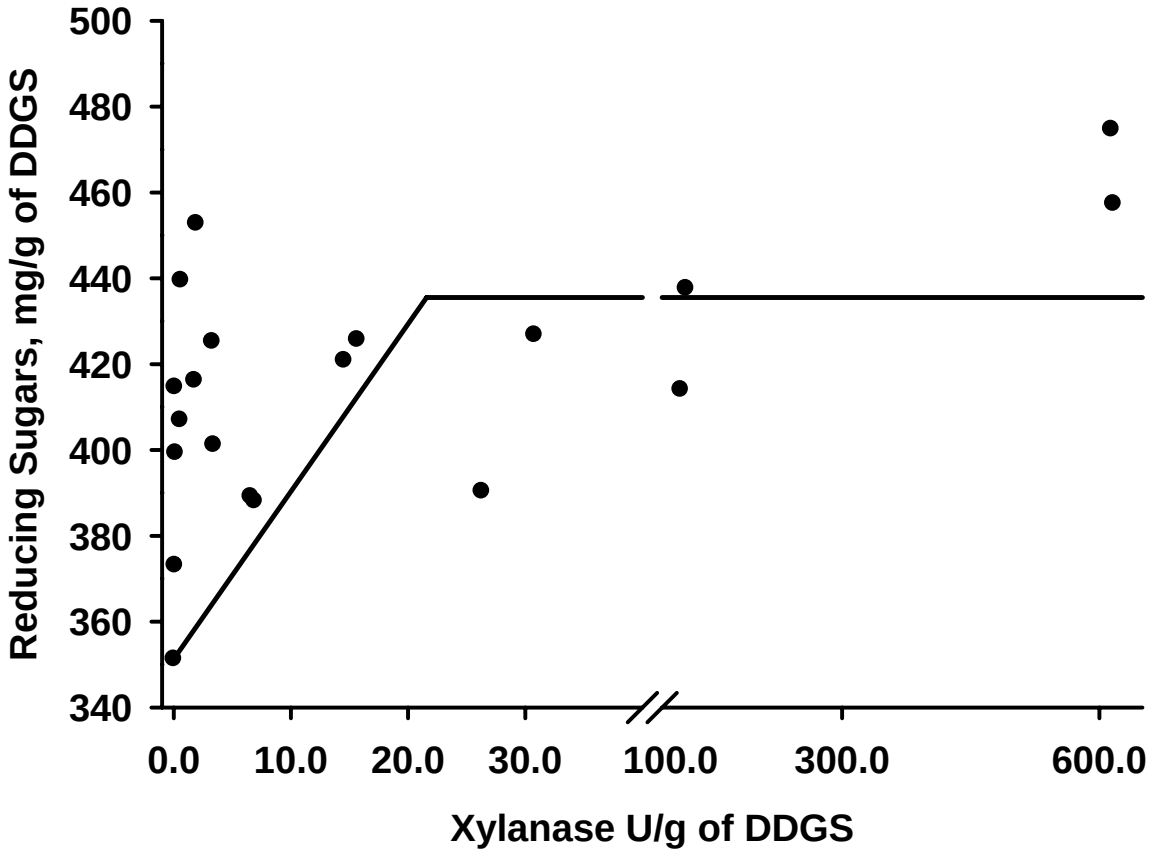

Figure 3.6. Effect of xylanase activity on the release of reducing sugars from distillers dried grains with solubles (DDGS). Increasing xylanase concentrations were incubated with DDGS at 39^{o}C for 15 min. Reducing sugars were determined using the Nelson-Somogyi procedure (Nelson 1944; Somogyi, 1952). A broken-line was fit to data to determine the break-point, which occurred at 21.58 U/g, releasing 435.5 mg/g of reducing sugars. The linear slope is 2.13, $r^2 = 0.40$.

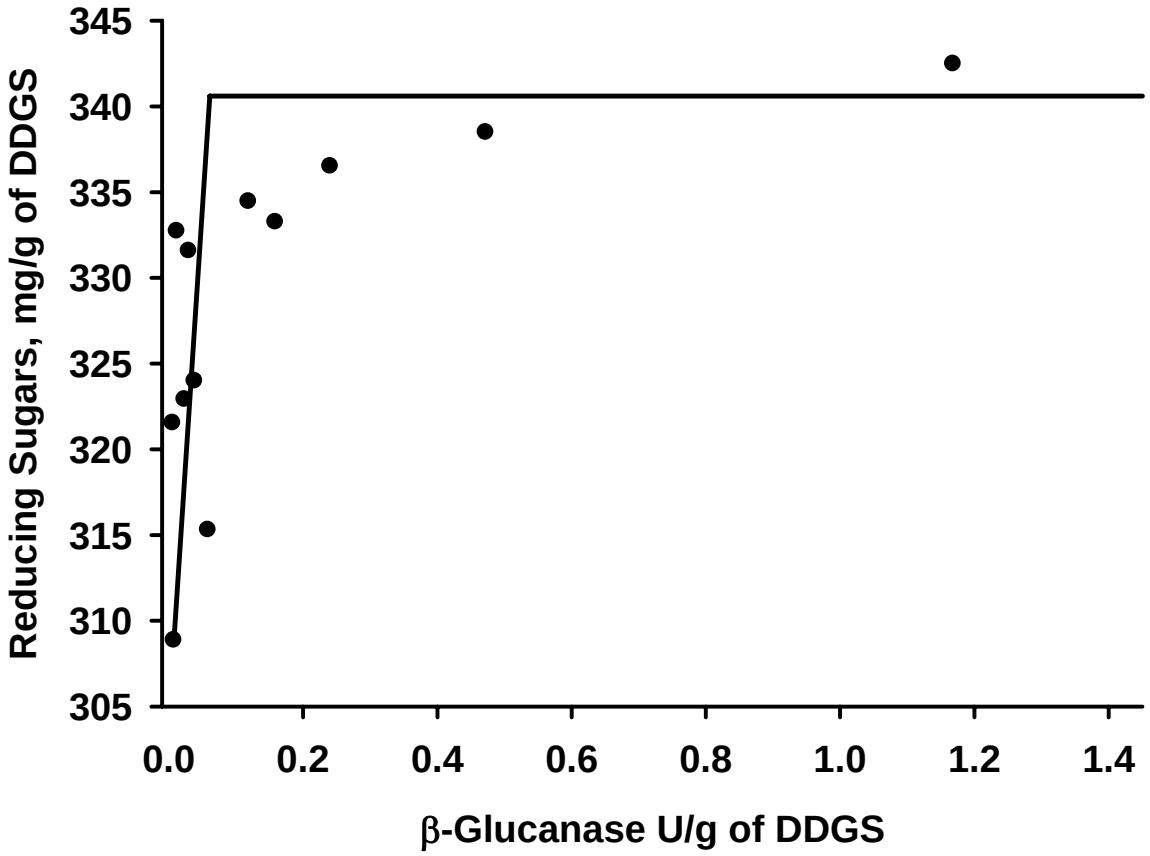

Figure 3.7. Effect of β-glucanase activity on the release of reducing sugars from distillers dried grains with solubles (DDGS). Increasing β-glucanase concentrations were incubated with DDGS at 39^{o}C for 15 min. Reducing sugars were determined using the Nelson-Somogyi procedure (Nelson 1944; Somogyi, 1952). A broken-line was fit to data to determine the break-point, which occurred at 0.06 U/g, releasing 340.6 mg/g of reducing sugars. The linear slope is 381, $r^2 = 0.67$.

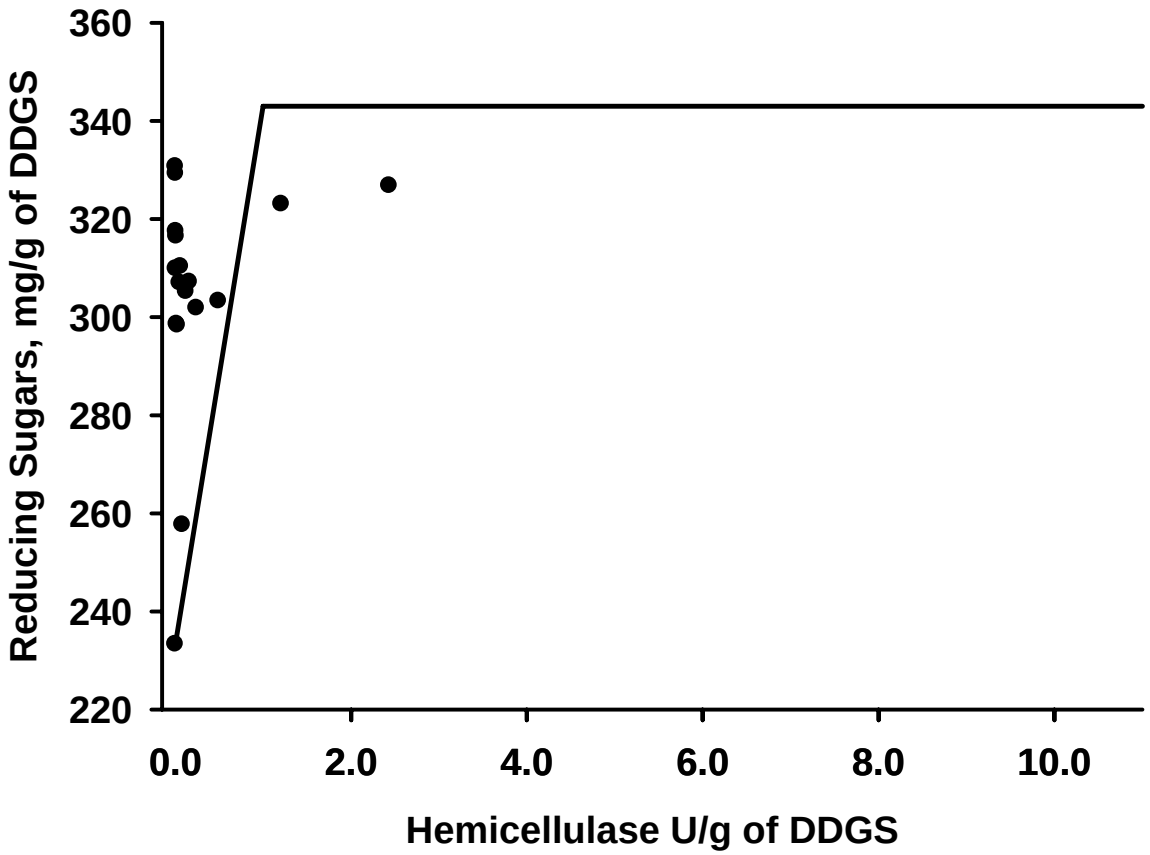

Figure 3.8. Effect of hemicellulase activity on the release of reducing sugars from distillers dried grains with solubles (DDGS). Increasing hemicellulase concentrations were incubated with DDGS at 39^{o}C for 15 min. Reducing sugars were determined using the Nelson-Somogyi procedure (Nelson 1944; Somogyi, 1952). A broken-line was fit to data to determine the break-point, which occurred at 2 U/g, releasing 343 mg/g of reducing sugars. The linear slope is 32.62, $r^2 = 0.39$.

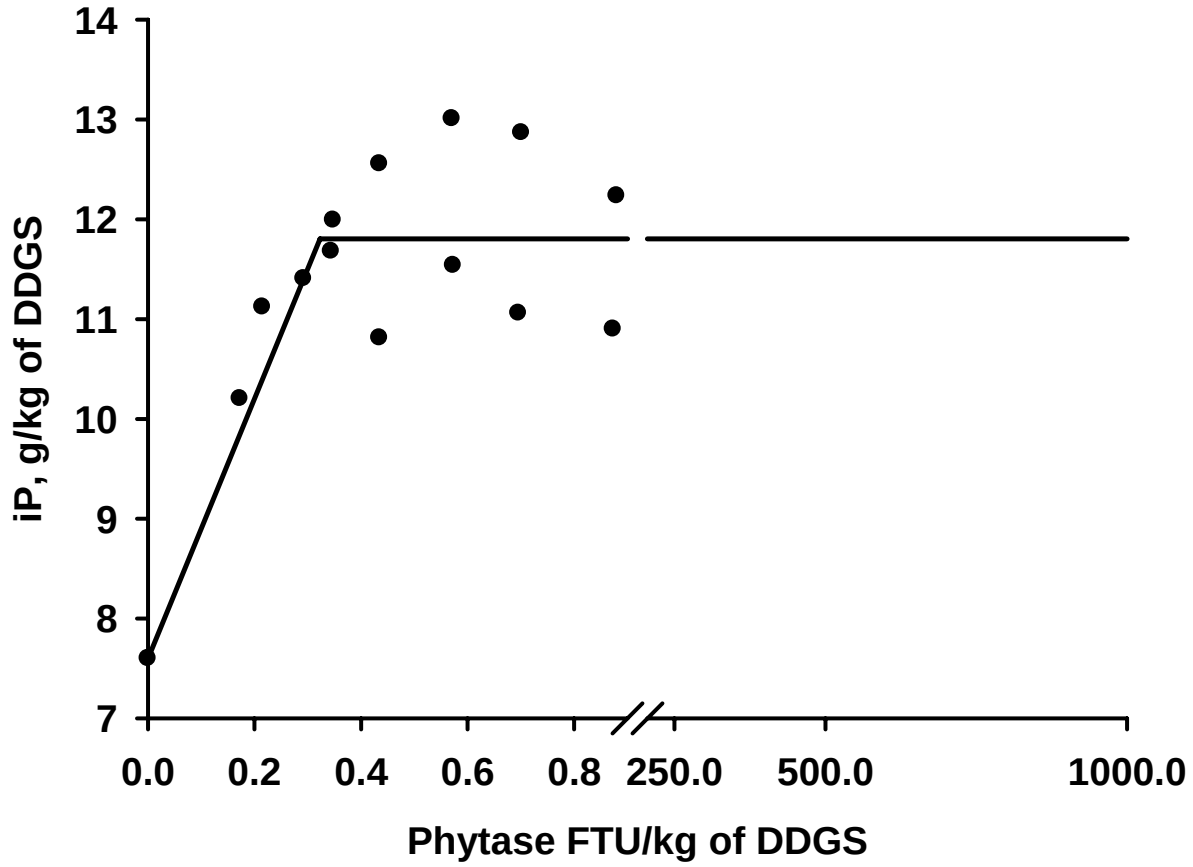

Figure 3.9. Effect of phytase activity on the release of inorganic phosphorus from distillers dried grains with solubles (DDGS). Increasing phytase concentrations were incubated with DDGS at 39°C for 15 min. Inorganic phosphorus (iP) was determined using the AOAC procedure (AOAC, 2005). A broken-line was fit to data to determine the break-point, which occurred at 0.32 FTU/kg, releasing 11.8 g/kg of inorganic phosphorus. The linear slope is 9.26, $r^2 = 0.91$.

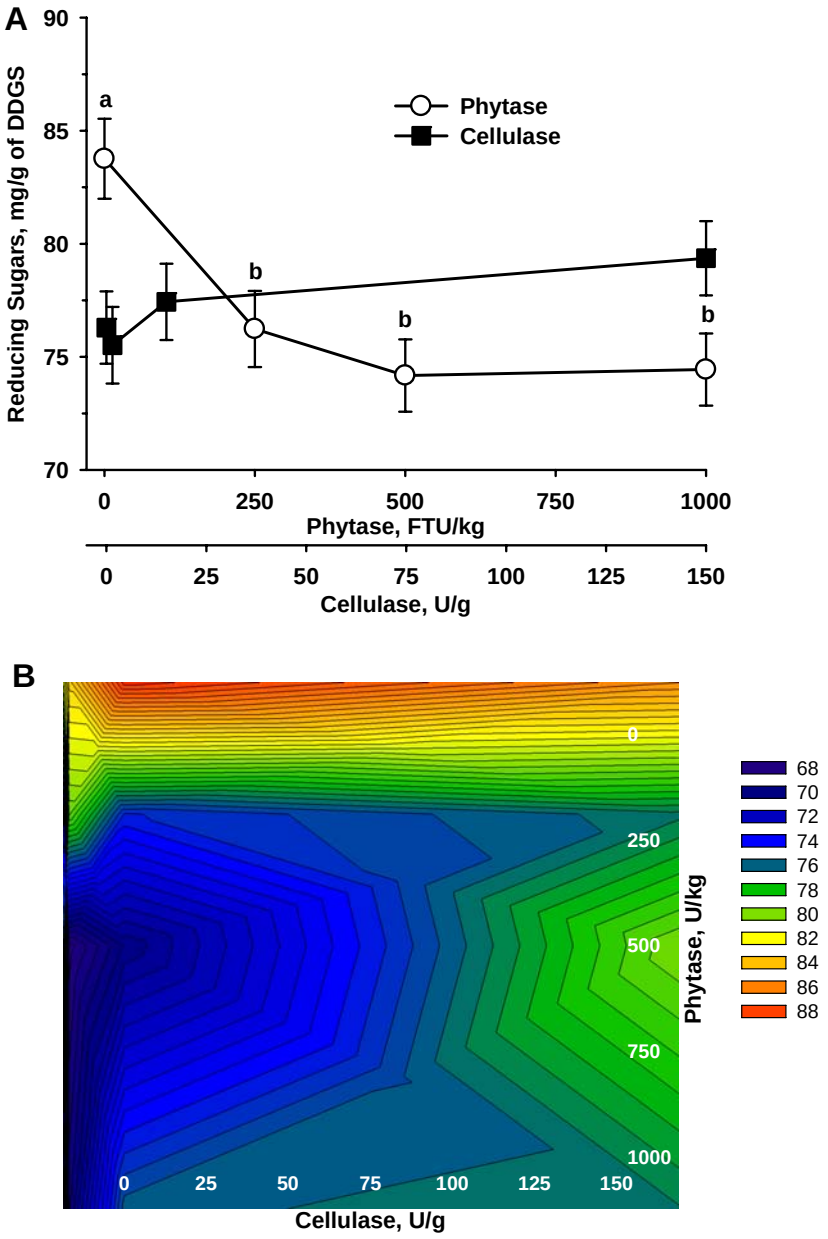

Figure 3.10. Effect of cellulase on its ability to release reducing sugars from distillers dried grains with solubles (DDGS), and the effect of phytase on the ability of cellulase to release reducing sugars during an *in vitro* digestion (A). Differing letters differ at $P < 0.003$. Surface analysis of the interaction of phytase and cellulase on reducing sugars released from DDGS during an *in vitro* digestion (B). Values are means ± SEM. n = 96.

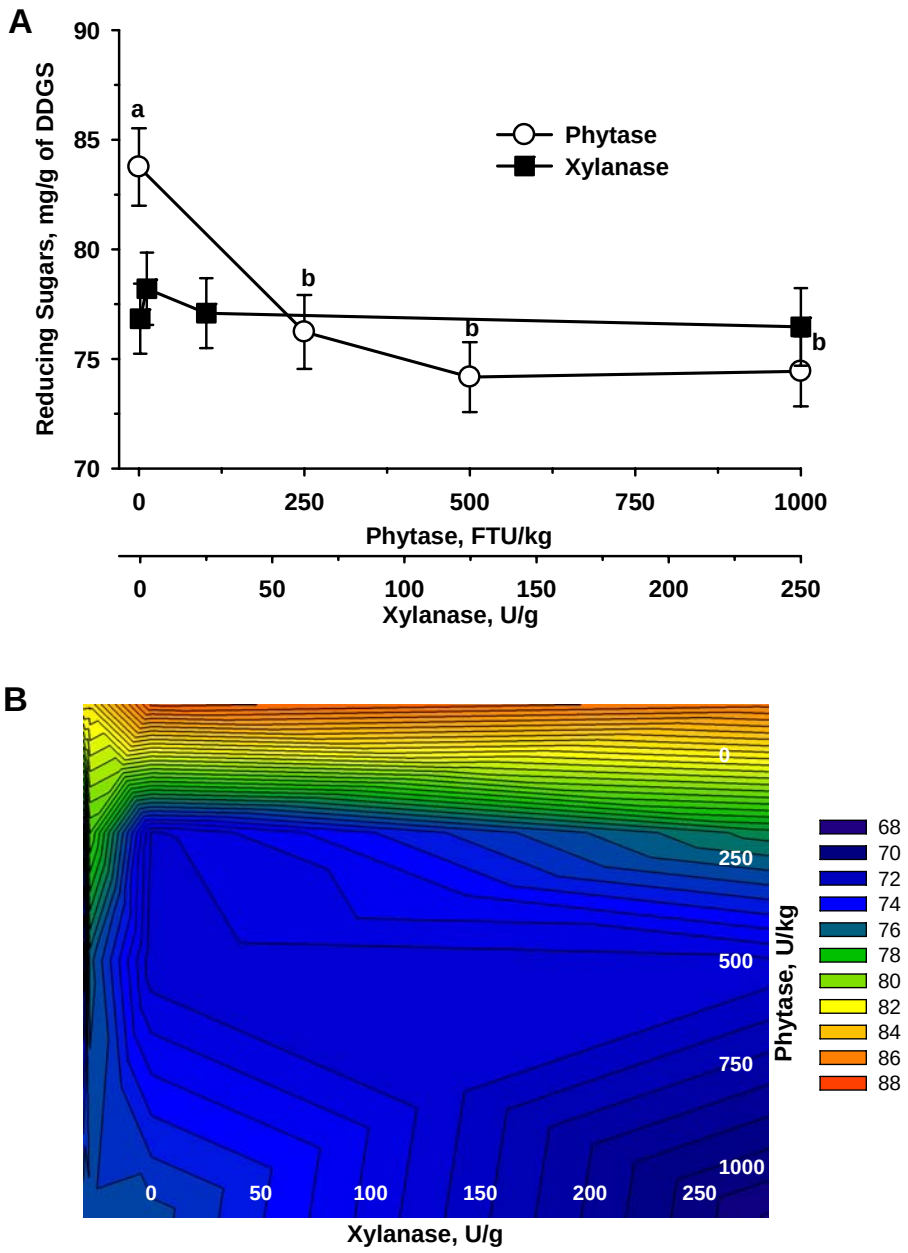

Figure 3.11. Effect of xylanase on its ability to release reducing sugars from distillers dried grains with solubles (DDGS), and the effect of phytase on the ability of xylanase to release reducing sugars during an *in vitro* digestion (A). Differing letters differ at $P < 0.003$. Surface analysis of the interaction of phytase and xylanase on reducing sugars released from DDGS during an *in vitro* digestion (B). Values are means ± SEM. n = 96.

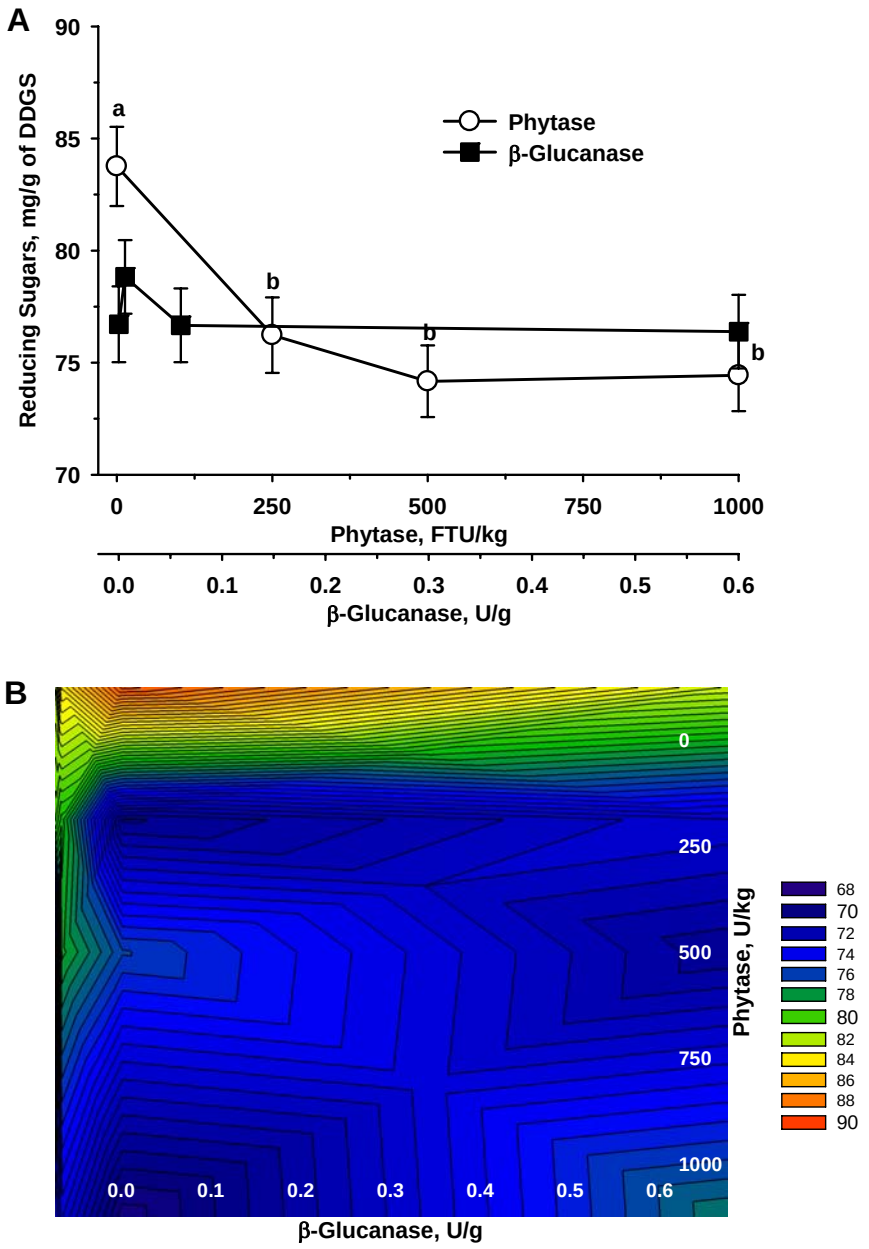

Figure 3.12. Effect of β-glucanase on its ability to release reducing sugars from distillers dried grains with solubles (DDGS), and the effect of phytase on the ability of β-glucanase to release reducing sugars during an *in vitro* digestion (A). Differing letters differ at $P < 0.003$. Surface analysis of the interaction of phytase and β-glucanase on reducing sugars released from DDGS during an *in vitro* digestion (B). Values are means ± SEM. n = 96.

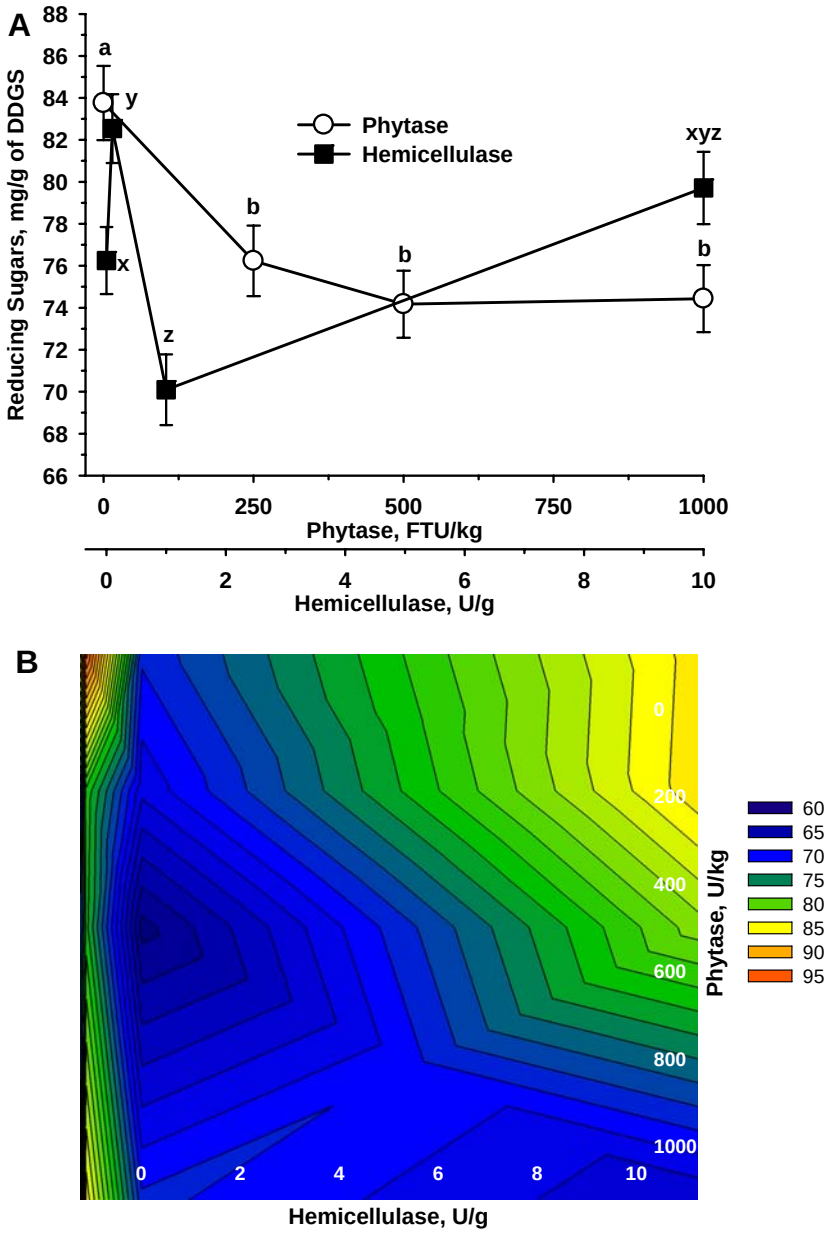

Figure 3.13. Effect of hemicellulase on its ability to release reducing sugars from distillers dried grains with solubles (DDGS), and the effect of phytase on the ability of hemicellulase to release reducing sugars during an *in vitro* digestion (A). Differing letters differ at $P < 0.003$ for phytase. Letters that differ for hemicellulase differ at $P < 0.008$. Surface analysis of the interaction of phytase and hemicellulase on reducing sugars released from DDGS during an *in vitro* digestion (B). Values are means ± SEM. n = 96.

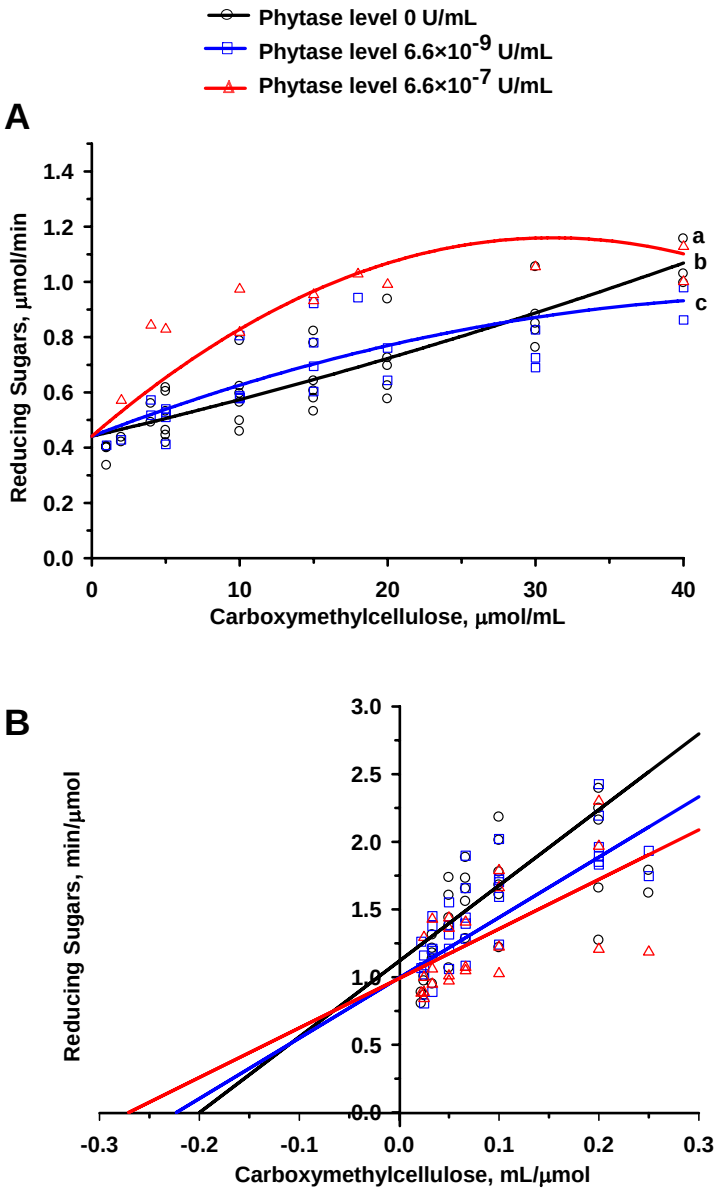

Figure 3.14. Effect of phytase on the ability of cellulase to release reducing sugars from carboxymethylcellulose. (A) Michaelis-Menten plot. The equation for phytase 0 U/mL is $y=-0.00008x^2 + 0.0125x + 0.4414$, $r^2=0.87$. The equation for phytase 6.6×10^{-9} U/mL is $y=-0.0002x^2 + 0.0205x + 0.4414$, $r^2=0.67$. The equation for phytase 6.6×10^{-7} U/mL is $y=-0.0007x^2 + 0.0461x + 0.4414$, $r^2=0.78$. Differing letters differ at $P < 0.03$. (B) Lineweaver-Burk plot. The equation for phytase 0 U/mL is $y= 5.6x + 1.12$. The equation for phytase 6.6×10^{-9} U/mL is $y= 4.46x + 0.99$. The equation for phytase 6.6×10^{-7} U/mL is $y= 3.66x + 0.99$. n = 6.

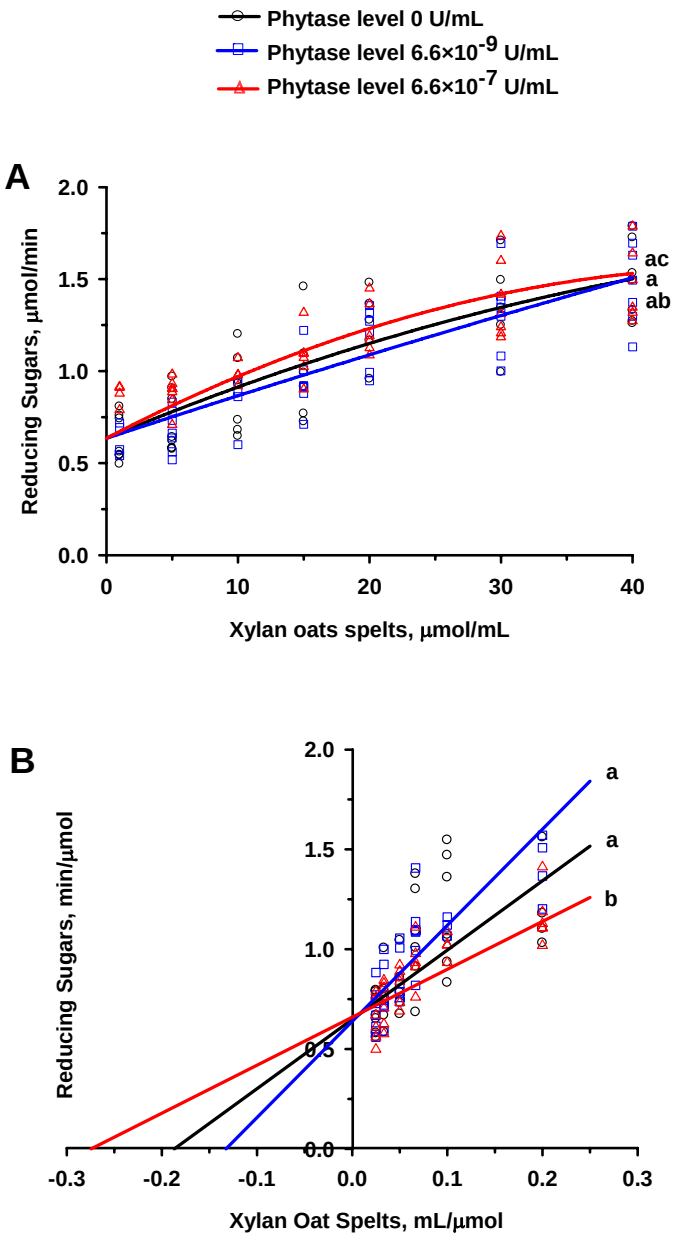

Figure 3.15. Effect of phytase on the ability of xylanase to release reducing sugars from xylan oats spelts. (A) Michaelis-Menten plot. The equation for phytase 0 U/mL is $y=-0.0002x^2 + 0.0299x + 0.6351$, $r^2= 0.71$. The equation for phytase 6.6×10^{-9} U/mL is $y=-0.00004x^2 + 0.0236x + 0.6351$, $r^2=0.77$. The equation for phytase 6.6×10^{-7} U/mL is $y=-0.004x^2 + 0.0373x + 0.6351$, $r^2=0.79$. Differing letters differ at $P < 0.09$. (B) Lineweaver-Burk plot. The equation for phytase 0 U/mL is $y= 3.47x + 0.65$. The equation for phytase 6.6×10^{-9} U/mL is $y= 4.81x + 0.64$. The equation for phytase 6.6×10^{-7} U/mL is $y= 2.40x + 0.66$. Differing letters differ at $P < 0.03$. n = 7.

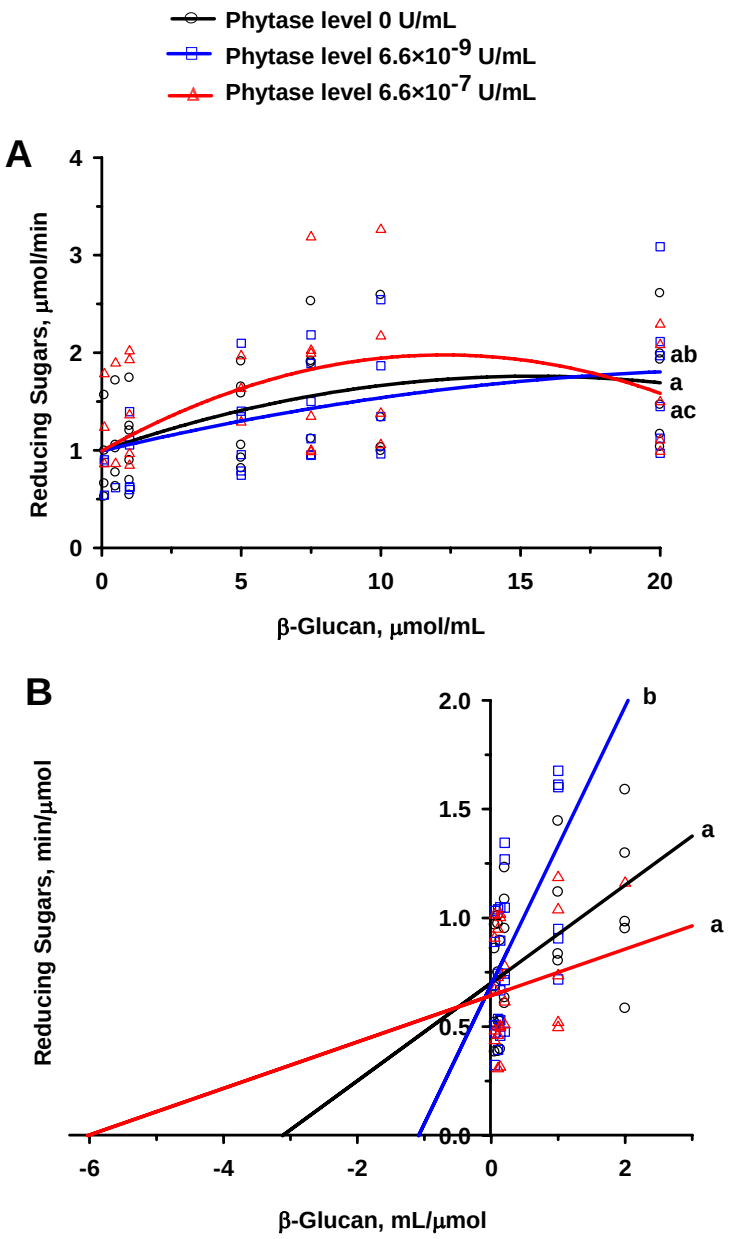

Figure 3.16. Effect of phytase on the ability of β-glucanase to release reducing sugars from β-glucan. (A) Michaelis-Menten plot. The equation for phytase 0 U/mL is $y=-0.003x^2 + 0.099x + 0.992$, $r^2= 0.27$. The equation for phytase 6.6×10^{-9} U/mL is $y=-0.001x^2 + 0.069x + 0.992$, $r^2=0.36$. The equation for phytase 6.6×10^{-7} U/mL is $y=-0.007x^2 + 0.161x + 0.992$, $r^2=0.10$. Differing letters differ at $P < 0.06$. (B) Lineweaver-Burk plot. The equation for phytase 0 U/mL is $y=0.22x + 0.70$. The equation for phytase 6.6×10^{-9} U/mL is $y=0.64x + 0.69$. The equation for phytase 6.6×10^{-7} U/mL is $y=0.11x + 0.64$. Differing letters differ at $P < 0.008$. n = 6.

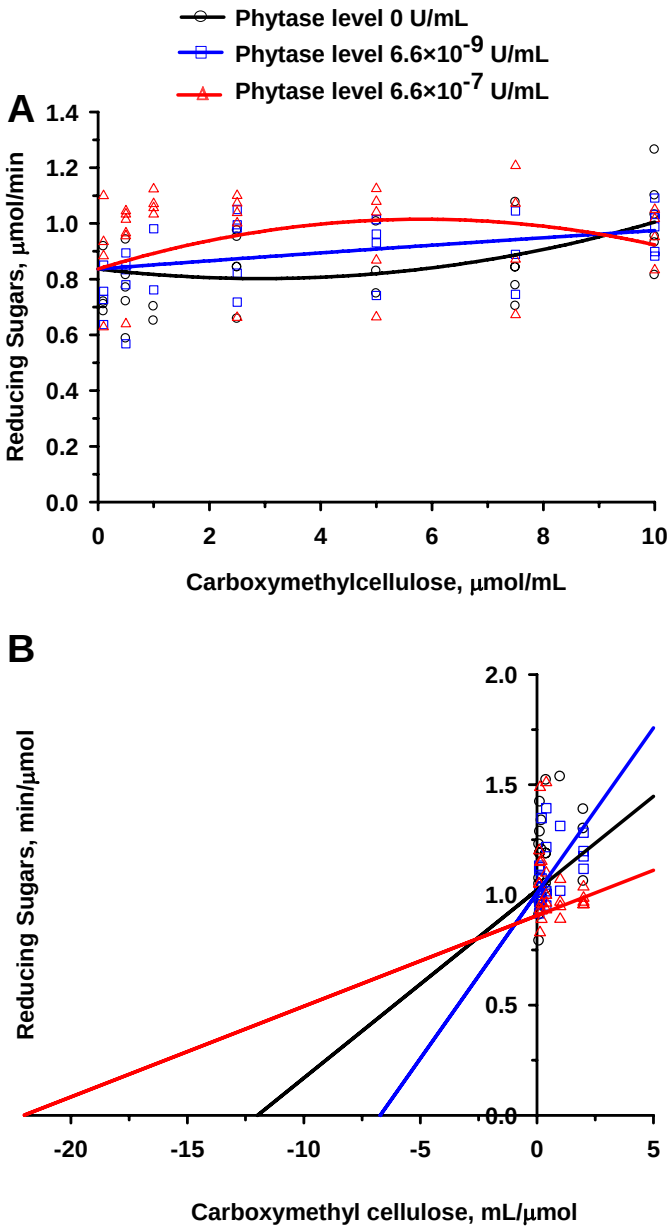

Figure 3.17. Effect of phytase on the ability of hemicellulase to release reducing sugars from carboxymethylcellulose. (A) Michaelis-Menten plot. The equation for phytase 0 U/mL is $y=0.004x^2 - 0.0024x + 0.837$, $r^2=0.34$. The equation for phytase 6.6×10^{-9} U/mL is $y=-0.0001x^2 + 0.0149x + 0.837$, $r^2=0.31$. The equation for phytase 6.6×10^{-7} U/mL is $y=-0.005x^2 + 0.061x + 0.0149$, $r^2=0.004$. (B) Lineweaver-Burk plot. The equation for phytase 0 U/mL is $y=0.09x + 1.02$. The equation for phytase 6.6×10^{-9} U/mL is $y=0.15x + 1.01$. The equation for phytase 6.6×10^{-7} U/mL is $y=0.04x + 0.91$. n = 7.

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