Effect of supplemental enzymes on the utilization of energy and phytate phosphorus in wheat by broiler chicks
by Ragothaman Ramachandran

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Animal Science
Montana State University

Abstract:
In mature cereal grains, legumes and oil seeds, the major portion of the total phosphorus is present in the form of phytic acid (phytate). A mixture of fiber degrading enzyme, proteolytic and phospholytic enzymes were supplemented to a wheat soybean meal based diets and were evaluated for their effectiveness in improving the production performance of Broiler chicks and their ability to release the phytate phosphorus from wheat and soybean meal and expose to endogenous phosphatases present in the gastrointestinal tract of the chick. Three experiments were conducted. In the first experiment three varieties of wheat were compared with and without enzyme at 0.1% level. In the second experiment four different levels of dietary phosphorus were compared with and without enzyme at 0.1% level. In the third experiment six levels of enzyme mixture were compared. Production performance of chicks fed diets supplemented with enzyme did not differ from those fed the control diets in all the three experiments. Level of dietary phosphorus had a positive correlation to fecal excretion of calcium and phosphorus and Bone Ash content. Enzyme mixture did not have any effect on phosphorus or calcium excretion. In order to release the phosphorus from phytin in a high fiber diets possibly warrants use of the phytase enzyme obtained from microbial sources apart from using fiber degrading enzyme so that the combined effect could increase effective phosphorus utilization from cereal grains and legumes and better utilization of fiber to improve performance in chicks.

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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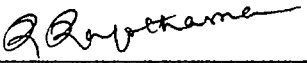
Signature 

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ABSTRACT

In mature cereal grains, legumes and oil seeds, the major portion of the total phosphorus is present in the form of phytic acid (phytate). A mixture of fiber degrading enzyme, proteolytic and phospholytic enzymes were supplemented to a wheat soybean meal based diets and were evaluated for their effectiveness in improving the production performance of Broiler chicks and their ability to release the phytate phosphorus from wheat and soybean meal and expose to endogenous phosphatases present in the gastrointestinal tract of the chick. Three experiments were conducted. In the first experiment three varieties of wheat were compared with and without enzyme at 0.1% level. In the second experiment four different levels of dietary phosphorus were compared with and without enzyme at 0.1% level. In the third experiment six levels of enzyme mixture were compared. Production performance of chicks fed diets supplemented with enzyme did not differ from those fed the control diets in all the three experiments. Level of dietary phosphorus had a positive correlation to fecal excretion of calcium and phosphorus and Bone Ash content. Enzyme mixture did not have any effect on phosphorus or calcium excretion. In order to release the phosphorus from phytin in a high fiber diets possibly warrants use of the phytase enzyme obtained from microbial sources apart from using fiber degrading enzyme so that the combined effect could increase effective phosphorus utilization from cereal grains and legumes and better utilization of fiber to improve performance in chicks.

INTRODUCTION

With increasing cost of production using conventional cereal grains like corn and sorghum in poultry diets, the poultry industry is turning to unconventional grains like barley, oats, rye, and wheat. The latter is often price-competitive with other small grains when bread quality standards are not met. Of the four small grains, wheat has the greatest feed potential due to its high starch and low fiber content. Although it is difficult to maximize growth performance with these grains, compared to highly digestible, high energy-dense diets based on corn, the use of supplemental enzymes may solve the digestibility problem to a certain extent.

In the Pacific Northwest and Great Plains states, barley and wheat are grown in large quantities while oats and rye are grown to a lesser extent. These grains are readily available in these areas of the country and are less expensive than corn, which must be shipped from midwestern markets. Wheat and barley are often used in poultry diets, but, wet, sticky fecal waste (litter quality) and inefficiency in performance of birds somewhat restricts their use, even when they are more cost effective than corn. Apart from performance problems, increased excretion of phosphorus and nitrogen in fecal waste from both poultry and pigs fed small grains has caused concern in areas of high population densities of animals and humans.

Certain enzymes have been tested in poultry feeds as a potential solution for litter quality and growth problems. Some success in performance has been achieved at the experimental level, although it remains to be seen if supplemental enzymes will work as well in commercial operations and in different production environments.

Phytate represents a potentially valuable source of phosphorus for monogastrics and removal of the phosphate groups deactivates its anti-nutrient activity. Phytate hydrolyzing enzymes, phytases, are produced in a variety of microorganisms and in limited amounts in monogastric animals (Power and Kahn, 1993). The concept of adding microbial phytase to the feedstuffs of monogastric animals to effect the release of phytate phosphorus was described over 25 years ago (Ware et al., 1967). Recent trials have shown that supplemental microbial phytase significantly improves phytate phosphorus availability in diets for monogastrics (Cromwell et al., 1994). Although the commercial production of microbial-derived phytase is now possible, technical difficulties still exist which preclude the widespread use of this enzyme in feedstuffs. The main area of concern for the most commonly used phytase (*Aspergillus ficuum* phytase) is its behavior under different pH conditions. The enzyme is unusual in that it has two pH optima; one at 2.5 and the other at 5.5. It is 48% less active at pH 2.5 than at pH 5.5 (Power and Kahn, 1993). The second major obstacle is that, while quite thermo-tolerant, pelleting at 70^0C reduced the enzyme's activity by 25%. Pelleting temperatures of 80^0C or greater led to unacceptable loss of activity (Schwarz and Hoppe, 1992).

LITERATURE REVIEW

Enzymes and Their Use in Poultry Diets

The Science of Enzymology

Historical Perspective. The science of enzymology began in the early 19th century with the discovery by Payen Persoz in 1833 that an alcohol precipitate of malt extract contained a thermolabile substance which converted starch into fermentable sugars. The enzyme was termed diastase because of its ability to separate soluble dextrins from insoluble starch grains. Several enzymes, including pepsin, polyphenol oxidase, peroxidase and invertase were identified in the middle and late 19th century. In 1884, Jokichi Takamine patented the first industrial application for an enzyme which he named "Taka-Diastase". This diastatic enzyme was derived from a mold, *Aspergillus oryzae*, that was grown on rice. Enzymology evolved at a relatively slow pace over the next few years. The term "enzyme" was first proposed by Kuhne in 1878 and Emil Fischer developed the concept of enzyme specificity in 1894. The studies of Fischer resulted in the famous "lock and key" analogy of enzyme substrate interaction which is illustrated in the following equation:

[Enzyme]+[Substrate] → [Enzyme-Substrate]=[Enzyme]+[Product]

With the acceptance of this concept, quantitative methods were developed for describing the action of enzymes. In 1913, Michaelis and Menton derived their famous mathematical expression which described quantitatively the kinetic behavior of the enzyme substrate complex (Lehninger, 1975).

Several research groups began purifying enzymes in the 1920's, although the chemical composition of these compounds remained unknown. In 1926 Sumner was the first to succeed in purifying and crystallizing an enzyme which released ammonia from urea. Sumner reported the enzyme (urease) to be a protein, but it was not until 1929 that this was acknowledged by the scientific community. Following Sumner's publication in 1926, many enzymes were crystallized and purified in the 1930's. In 1959, Koshland introduced the "induced fit" concept of enzyme substrate combination. The "induced fit" theory retained the Fischer's concept of stereospecific conformation between enzyme and substrate, but rejected the idea that the binding site on the enzyme was a rigid structure. Koshland proposed that the presence of substrate near the active site could cause changes which would bring about a closer fit between substrate and enzyme (Lehninger, 1975).

Enzyme Classification. Enzymes are proteins which are natural catalysts produced by living cells (Enari, 1983). Underkofler et al. (1958) proposed the following definition of an enzyme: "Enzymes are biocatalysts, produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic process of the cells. Enzymes are highly specific in their action on substrates and often many different enzymes are required to bring about, by

concerted action, a sequence of metabolic reactions performed by the living cell. All enzymes which have been purified are protein in nature, and may or may not possess a non-protein prosthetic group".

Enzymes are named and classified according to their specificity for substrate and resulting reactions. The letters "ase" are added to end of the name of the substrate upon which the enzyme acts. There are six groups into which enzymes are classified, depending upon the reaction catalyzed. These are oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Each classification is further subdivided until enzymes are identified by a chemically meaningful six figure code. Enzymes are also classified as "endo" or "exo", referring to the way the enzyme attacks the substrate molecule. Endo- enzymes attack the substrate at the interior bonds, while exo- enzymes approach the substrate from one or the other ends of the molecule (Dixon and Webb, 1964).

Enzyme use in industry and agriculture

Historical Perspective

The first serious attempts to use enzymes for industrial purposes in the early 1900's met with limited success. This was due largely to a lack of understanding of enzyme activity. As knowledge progressed with the characterization of enzymes and an understanding of enzyme kinetics, applications were developed for a variety of industrial processes. The majority of enzymes currently used in industry i.e., pectinases, lipases, carbohydrases, etc. may be described as hydrolytic depolymerases. The inclusion of proteases and amylases in detergent preparations

is the single most significant industrial application of enzymes to date. Carbohydrases, especially amylases and pectinases, account for a significant portion of the remaining market. Glucose isomerase is one of the few non-depolymerases that is widely used in industry (Sears and Walsh, 1993). A growing use of enzymes is their application to analytical biochemistry as in the analysis for extractable and nonextractable β-glucans developed by McCleary and Glennie-Holmes (1985).

Various enzymatic preparations have been used medically as digestive aides, such as lactase for lactose intolerance in people. The therapeutic benefit of using enzymes for such purposes in humans has been long recognized (Sears and Walsh, 1993). Similarly, the concept of using microbial-derived enzymes in animal feeds to improve the nutritive value of low quality feeds and the performance of animals dates back over thirty years. Feeding of partially sprouted barley and rye was found to increase the nutritive value of these grains in early 1950's. The improvements were possibly due to the activation of endogenous native enzymes present in the seeds. Two of the early attempts in enzyme application to improve animal feed were made by Jensen et al. (1957) and Burnett (1962), who added crude amylase and protease preparations to chicken diets. These preparations were later found to contain β-glucanase activity (Rickes et al., 1962). Ware et al.(1967) and Nelson et al. (1968) demonstrated the effectiveness of microbial produced phytase for increasing the utilization of phosphorus from plant sources by chickens. As with the industrial application of enzymes, several factors contributed to failure or low overall effectiveness of enzymes in these earlier attempts to use them in animal

feeds. These factors included the use of crude enzyme preparations, little understanding of animal physiology and feedstuff composition, as well as little knowledge of optimal pH for maximum enzymatic activity, and the mode and kinetics of the reactions. Confusion also developed because of differences in response to supplemental enzymes by different experimental animals.

Although a great amount of research has been performed, it is only recently that the true potential of incorporating enzymes into animal diets has been appreciated. Goals of enzyme supplementation of animal diets are to remove or destroy anti-nutritive factors, enhance overall digestibility, render certain nutrients biologically available, and reduce the pollution impact of animal excreta (Inborr, 1989). Since the early beginnings, applied research on the use of enzymes in diets for poultry and other monogastric animals has greatly intensified.

Substrate Targets in Poultry Diets

Cereal grains make up the greatest portion (70 to 80%) of poultry diets in North America, with the bulk of the rest being soybean meal and other high protein feedstuffs (USDA, 1992). In the Northern Great Plains and Pacific Northwest states, wheat and barley are the major cereal grains. Smaller amounts of rye, triticale and oats are also grown. These crops are referred to as small grains, as opposed to corn or maize. Although considerable amounts of corn are used in poultry diets in this geographical area, it must be imported at substantial cost from the midwestern and southern states.

The majority of the components in small grain cereals, as with corn, are

carbohydrates (>80%) which are primarily starch and nonstarch polysaccharide (NSP) with a small portion (1 to 3%) of free sugars (Henry, 1985). Of the carbohydrates in small grain cereals, 75% to 90% is composed of starch, with nonstarch polysaccharides (NSP) making up from 10 to 25% of the total. Although cereal grains provide a significant portion of protein to poultry diets, the major nutrient furnished by cereal grains is energy. Dietary energy is primarily derived from starch with smaller amounts coming from lipids, nonessential amino acids, free sugars and NSP. Most of the NSP is not digested by monogastrics because they lack the necessary enzymes in the gastrointestinal tract to do so. That portion of NSP that is digested furnishes only a small percentage of the total energy released from the cereal grains. This is accomplished primarily in the caecum and large intestine by microbial fermentation. In many instances, the soluble portion of NSP in cereal grains increases the viscosity of the digesta thereby restricting nutrient absorption not only affecting the absorption of basic nutrients such as glucose, fat and protein (Fengler and Marquardt, 1988b; Wang, 1992), but the utilization of calcium, phosphorus and zinc (Southgate, 1987; Gordon, 1990). Under these conditions soluble NSP are classified as antinutrients.

Two forms of starch are found in cereal grains, amylose and amylopectin. Amylose is a linear chain of glucose units linked via α-1,4 glycosidic bonds. Amylopectin, on the other hand, is highly branched. Successive glucose units are linked via α-1,4 glycosidic bonds with branching points linked with α-1,6 glycosidic bonds. The NSP of small grain cereals is composed principally of cellulose, mixed

linked (1-3),(1-4)-β-glucans, commonly referred to as β-glucans, and pentosans (arabinoxylans). Covered or hulled barley and oats are the only small grains that contain significant amounts of cellulose. Most of the cellulose in barley and oats is in the hulls, although small amounts are found in the aleurone and endosperm cell walls as is the case with wheat, rye, and triticale. Naked or hulless barley is similar to wheat and rye in cellulose content. Although not a carbohydrate, lignin is generally included as part of NSP because of its relationship with cellulose. β-glucans are the major NSP in the endosperm and cell walls of barley and oats. In barley, β-glucans are found in both the aleurone and endosperm cell walls but in greater concentrations in the latter. The concentration of β-glucans in barley as well as their molecular weights vary with genotype (Bengtsson et al., 1990; Xue et al., 1991). In contrast to barley, β-glucans in oats are concentrated in the outer portion of the kernel with considerably less in the endosperm cell walls (Bacic and Stone, 1981). About one-third of the endosperm cell wall NSP in barley is arabinoxylan. In wheat, rye, and triticale, arabinoxylans are the major NSP with only small amounts of β-glucans (Mares and Stone, 1973; Ciacco and D'Appolonia, 1982; Henry, 1985). As with β-glucans in barley and oats, the arabinoxylans of wheat, rye, and triticale are located in the aleurone and endosperm cell walls (D'Appolonia and MacArthur, 1976). Two types of pentosans have been described in rye (Bengtsson and Åman, 1990). Pentosan-1 is characterized by single arabinose units linked β-(1-3) to the primary xylan β-(1-4) chain. A second fraction, Pentosan-2 was isolated and found to contain sequential xylose residues (4-5) that were doubly

branched at the -2 and -3 positions.

Phytic acid comprises 1.0 to 1.5% of the content of cereal grains and typically represents from 50 to 80% of total seed phosphorus (Raboy, 1990). Phytic acid is principally deposited as discrete globular inclusions in single-membrane storage microbodies referred to as protein bodies (Pernollet, 1978; Lott, 1984). In wheat and barley, the protein bodies contain proteinaceous matrix, which surrounds phytate-rich globoid crystals (Jacobsen et al., 1971; Raboy, 1990). Most of the phytate (approximately 90%) in wheat (and possibly in barley) is found in the aleurone, with about 10% found in the germ (embryo and scutellum). In maize, the reverse occurs, with nearly 90% of the phytate localized in the germ and 10% in the aleurone (O'Dell et al., 1972).

Enzymes for Substrate Targets in Poultry Diets

The major starch degrading enzymes are α- and β-amylases, glucoamylases, pullulanases, and isoamylases (Lehninger, 1975). α-Amylase is an endo-enzyme which splits α-1,4-glucosidic bonds, except for that of maltose, in an apparently random fashion. β-Amylase, an exo-enzyme, effects the successive removals of maltose units from the nonreducing ends of glucose chains in starch. Neither α- nor β-amylase exhibits activity against α-(1-6) bonds or against β-(1-4) bonds. Glucoamylase, also known as amyloglucosidase, is an exoenzyme catalyzing the sequential removal of glucose residues from the nonreducing ends of the glucose chains, splitting both α-(1-4) bonds and α-(1-6) bonds. Pullulanase and isoamylase are endoenzymes capable of hydrolyzing α-(1-6) bonds found at the branching points

in amylopectin. Endoenzymes that catalyze the hydrolysis of α-(1-6) bonds are called debranching enzymes. Under normal conditions, starch in cereal grains is almost completely converted to glucose for absorption by the digestive system.

Cleavage of the β-(1-4) glycosidic bonds in cellulose requires a combination of cellulases rather than any specific single enzyme (Sears and Walsh, 1993). Additionally, lignin encrustation renders access to the glycosidic bonds in cellulose by the enzymes difficult, if not impossible. The most important enzymes that depolymerize the cell wall β-glucans are the (1-3),(1-4) β-glucan 4 glucohydrolases. Two (1-3),(1-4)-β-glucan endohydrolases have been purified from extracts of germinated barley. The optimum pH for these β-endoglucanases is 4.7, thus they are not entirely suited to the gastric or intestinal pH of monogastric animals. The complete depolymerization of cell wall arabinoxylans in barley is accomplished by the concerted action of endo- and exoxylanases, α-arabinofuranosidase, and possibly xylobiase (Preece and MacDougall, 1958).

The enzyme phytase (mesoinositol hexaphosphate phosphohydrolase) acts on phytate to yield inositol and orthophosphates (Reddy et al., 1982). Phytase occurs in varying amounts in plants and is produced by yeast, fungi, and bacteria (Patwardhan, 1937). While the flora of ruminants are well known to produce potent phytases (Reid et al., 1947) it is less commonly known that phytate hydrolyzing enzymes exist in the intestines of most monogastric animals (Spitzer and Phillips, 1945; Nelson, 1967; Davies et al., 1970; Davies and Motzok, 1972; Pointillart et al., 1984) including humans (Bitar and Reinhold, 1972). However, these enzymes are

extremely weak and are inhibited by nutrients such as calcium (Power and Kahn, 1993). Cereal grain seeds have active phytases, but these enzymes have pH optima ranging from 5.0 to 7.5, making them apparently unsuitable for use at low stomach pH values (Power and Kahn, 1993). However, earlier reports indicate that plant phytases retain their activity under *in vitro* conditions similar to that of the chick intestine (Singsen et al., 1944; Courtois, 1945; Møllgaard, 1946).

Present status of Enzyme applications in poultry diets

β-Glucanases and pentosanases are the two major categories of enzyme supplements that have been extensively researched and are currently used commercially in poultry feeding systems with the intent to improve nutrient utilization, litter quality and (or) egg cleanliness. Of the two enzymes, β-glucanase has proven in many experiments to be dramatically effective (Hesselman et al., 1982). Pentosanases have shown variable results (Annison, 1992). Poultry research reports on the specific effects of other enzyme categories such as cellulases, pectinases, proteases, lipases, and amylases are limited. Most enzyme additives for animal feeds are crude preparations and generally exhibit activity towards a range of substrates (Campbell and Bedford, 1992). Commercial enzyme products are often blends of two or more of the enzyme groups and are referred to as "enzyme cocktails" (H. Graham, personal communication).

Little scientific evidence supports the use of amylases in poultry diets, even though the concept of improving starch digestibility for genetically improved, fast-growing birds is intriguing. The pioneering studies of Willingham et al. (1959)

showed that crystalline amylase was ineffective in improving barley diets for poultry and that the beneficial effects he reported were due to β-glucanase present in the crude enzyme mixture. Amylase excretion in the small intestine of poultry is obviously at such a level that starches are well digested and utilized (Moran, 1982). It has been postulated by a number of authors (see review by Campbell and Bedford, 1992) that very young birds could benefit most from amylases and other enzyme supplements in the feeds. Such is the case with β-glucanase and pentosanases, but scientific evidence with other enzyme systems to support this contention is limited (Campbell and Bedford, 1992).

Equally intriguing as the idea of using amylases is the concept of using cellulase to enhance energy levels in feedstuffs high in insoluble fiber, such as hulled barley, oats or byproducts of the brewing and distilling industries. The process of enzymatic hydrolysis of cellulose is extremely complex, however, involving many different cellulase activities. Additionally, cellulose is rarely found in pure form in nature (cotton being the exception), especially in feedstuffs.

Currently, phytase is the only enzyme that has the potential to dramatically improve nutrient utilization in poultry feeds at the same magnitude as that of glucanases and pentosanases. Given a maximum effect on phytate, the resulting improvements could exceed that of these two carbohydrases. Whereas the problems of β-glucans are limited to barley and oats, and pentosans to rye and to a lesser extent wheat, phytate is universally present in *all* plant material. As previously noted, phytate in feedstuffs represents a major source of phosphorus for meeting the

requirements for growth and bone development, but as such is almost entirely unavailable for poultry. The fact that phytate is an antinutrient, in that it irreversibly chelates divalent cations and interferes with amino acid absorption in the gastrointestinal tract of birds as well as other monogastrics, is sufficient cause to attempt to remove it from feedstuffs. Additionally, the fecal excretion of phytate phosphorus and chelated minerals is a major source of soil and water pollution when wastes are applied to farm land. Given these three reasons, improved utilization of plant phosphorus, removal of an antinutrient and the reduction of pollution, the successful utilization of phytase should surpass the overall benefits of any other single enzyme or enzyme system used in feeding regimens for monogastric animals, including poultry.

The following is a review of the pertinent early research and current reports on the application of glucanase, pentosanase and phytase to poultry diets.

β-glucanase

Supplementation of barley diets with glucanase has been shown to be effective in improving growth rate and feed efficiency of poultry. The greatest benefits have been shown in young broiler chicks (Elwinger and Saterby, 1987), although feeding trials to market weight have also demonstrated benefits in older birds (Campbell et al., 1984; Classen et al., 1988). As noted previously, in early work with carbohydrases, enzymes utilized were in crude mixtures. Rickes et al. (1962) obtained a purified glucanase from the enzyme mixture fed earlier by Jensen et al.(1957); they concluded that β-glucanase was responsible for improvement in

performance of the birds. Gohl et al. (1978) reported that β-glucanase or water treatment (mixing with warm water for 2 hours followed by drying) did not significantly influence the nutritional value of medium viscosity barley. However, when applied to high viscosity barley, β-glucanase or water treatment improved litter quality as well as performance of the birds. Hesselmann et al. (1981) showed that β-glucanase supplementation as a dry powder in the feed or drinking water of broiler chicks improved feed consumption, weight gain, and feed efficiency up to 21 days of age. Dry matter of excreta was increased and cage cleanliness was improved when the enzyme was consumed. This and later studies by Hesselmann et al.(1982; 1986) confirmed earlier findings of Rickes et al. (1962) that β-glucanase was the active enzyme in improving the growth rate and feed efficiency of broiler chicks.

Response to dietary β-glucanase is not uniform among barleys. Early reports alluded to differences between "Western" and "Eastern" barley (Willingham et al 1960). Western barley gave poorer initial growth of broilers, but a larger response to dietary enzyme inclusion. This difference was attributed by these authors to possibly higher endogenous enzyme (β-glucanase) levels found in Eastern barley. Burnett (1966) found that Australian barley had lower endogenous β-glucanase levels than Irish barley which corresponded to their feeding value. However, the pH optima of endogenous β-glucanases in the barley kernel precludes very little if any benefit from these enzymes under normal feeding conditions. Absolute viscosity and β-glucan levels of barley are affected by both genotype and environment (Aastrup, 1979; Hesselman and Thomke, 1982; Hockett et al., 1987; Newman and

Newman, 1987;1988). Barleys having higher levels of β-glucans always show greater response to glucanase supplementation as measured by improved chick performance (Newman and Newman, 1987; 1988; Classen et al., 1988; Campbell et al., 1989). Although studied much less extensively than barley, oats appear to behave similarly in regard to β-glucan content and β-glucanase supplementation (Elwinger and Saterby, 1987; Pettersson et al., 1987; Cave et al., 1990).

Several studies reported improvement in the absorption of fat, starch, nitrogen, and amino acids by chicks fed enzyme treated barley (Classen et al., 1988; Hesselman and Åman, 1986; Edney et al., 1989; Rotter et al., 1989; Wang, 1992). The improvement in nutrient digestibility in barley is believed to be due to the reduction of digesta viscosity by disruption of the β-glucan molecule. Complete conversion of β-glucan to glucose by β-glucanase would theoretically increase the metabolizable energy of barley or oats; however, most researchers conclude that the major effect is due to the reduced digesta viscosity.

Pentosanases

Halpin et al. (1936) concluded that rye was unsuitable for poultry because of reduced feed consumption and poor growth which was accompanied by sticky droppings. Similar results were found in later studies reported by Wieringa (1967) and Moran et al. (1969). It was then confirmed that nutrient utilization was depressed in chicks fed rye based diets (Misir and Marquardt, 1978a,b,c,d; Marquardt et al., 1979; Lee and Campbell, 1983) and the resulting performance was severely depressed.

Studies by Marquardt et al. (1979) and Antoniou et al. (1980) revealed that the depression of nutrient digestion, especially that of saturated fat, was due to a nonspecific antinutritional factor in rye. This factor was found to be concentrated in a water extractable fraction and it was hypothesized to be a water soluble portion of the pentosans (Fernandez et al., 1973a; Antoniou and Marquardt, 1981. Fractionation studies by Antoniou et al. (1981) indicated that the fraction causing nutrient depression was water soluble and was in fact rich in pentosans. Reports of Fengler and Marquardt (1988a,b) confirmed these findings. *In vitro* studies by Fengler and Marquardt (1988b) demonstrated that a pentosan-rich fraction extracted from rye impeded the dialysis of three different salts and glucose. Digestion of the pentosan with a crude extract of *Trichoderma viride* eliminated the viscosity of the solution and the dialysis rate was normalized. These authors further demonstrated that nearly all of the antinutritive activity of rye, as assessed by fat retention, was associated with the pentosan rich isolate. The causative factor of sticky droppings in poultry consuming barley was determined to be β-glucans which cause excessive losses of fat (Wang, 1992). The similar problem with sticky droppings from poultry fed rye diets was eliminated by supplemental β-glucanase and xylanase (Pettersson and Åman, 1989). These studies and that of GrootWassink et al. (1989), who fed a crude arabinoxylanase preparation to broiler chicks, confirmed that the antinutritive factor in rye grain is a water soluble pentosan. Further, these reports confirm the efficacy of pentosanase enzymes for improving the nutritive value of poultry diets based on rye.

The pentosans of wheat have also been implicated as antinutritive factors for poultry. Choct and Annison (1990) reported that addition of isolated arabinoxylans to broiler chick diets caused a depression in apparent metabolizable energy (AME) and growth. It has also been demonstrated that glycanase (glucanase + xylanase) supplementation of wheat-based broiler chick diets is beneficial (Inborr and Graham, 1991), indicating that wheat NSP are deleterious to broiler chick performance. A recent study showed a strong negative correlation between wheat (Australian) AME values and the level of water soluble NSP, which are predominately arabinoxylans (Annison, 1991). A later report by this author provided further evidence that cell wall material of wheat possesses antinutritive activity which may be reduced by supplementation of diets with glycanase preparations (Annison, 1992). In this study, supplemental enzymes raised the AME of wheat from 14.26 MJ/kg to 15.2 to 15.75 MJ/kg. From studies such as these it may be possible at some future date to predict nutritive value of wheat based on viscosity extracts which are directly related to intestinal viscosity created by pentosans (Choct and Annison, 1992) as β-glucans in barley (Campbell et al., 1989, Rotter et al., 1989; Wang et al., 1992).

Phytase

Phytate represents a potentially valuable source of phosphorus for monogastric animals because small grain cereals contain relatively high levels of this compound. Additionally, soybean meal, the most commonly used source of supplemental protein in poultry diets in North America, contains even higher levels

of phytate than cereal grains (Raboy, 1990). Phytate phosphorus, however, must be released enzymatically in order to become available for absorption and utilization in animals. As noted previously, low levels of active phytase occur in gastrointestinal tracts of humans, poultry, and other animals. Wheat, rye, triticale, and their byproducts, and to a lesser extent barley, were fairly rich sources of phytase (McCance and Widdowson, 1942; Møllgaard, 1946); whereas, oats, corn, and soybean meal contained little or no phytase. Recently, Bos (1990) reported that wheat, triticale, rye and wheat grits contained high levels of phytase while barley contained moderate amounts. Pigs fed wheat- or barley-based diets require less supplemental phosphorus than those fed corn- or grain sorghum-based diets to maximize performance and bone mineralization (Cromwell et al., 1972a, 1974; 1979). These results could have been influenced by the higher total levels of phosphorus in these grains compared to that in corn and grain sorghum. However, other researchers have reported that the availability of wheat or triticale phosphorus is higher than that of corn for pigs (Pointillart et al., 1984, 1987) and poultry (Sauveur, 1989). A review of literature by Nelson (1967) on the utilization of phytate phosphorus by poultry cited widely varying views of researchers up to that date on the availability of plant phosphorus. The preponderance of data presented by Nelson (1967) indicated that it is questionable whether any portion of phytate phosphorus should be considered available for utilization by poultry.

Phytate interference with mineral absorption is a well documented fact in humans (McCance and Widdowson, 1942; Reinhold, 1971; Reinhold et al.,1973;

Reinhold et al., 1976; Faramarz et al., 1977) and domestic animals (Mellanby, 1949; Vohra and Kratzer, 1966; Reinhold et al., 1974; Southgate, 1987). Phytate can form undigestible chelates with metallic divalent cations such as zinc, magnesium and iron (Graf, 1986). The severity of phytate interference on mineral absorption depends upon several factors: the presence and activities of endogenous seed and yeast (in leavened breads) phytases, type of phytate salt, i.e., magnesium, potassium etc., dietary calcium level, total dietary fiber level and type (soluble or insoluble), presence of vitamin D, species and age of animals (Reinhold et al., 1976; Farah et al., 1984; Ballam et al., 1985). For several years, it has generally been accepted among nutritionists that phytase has the potential to enhance phosphorus availability when added to the diets of nonruminants. Recent studies with pigs have clearly demonstrated enhancement (Simons et al., 1990; Jongbloed et al., 1990; Ketaren et al., 1991; Mroz et al., 1991; Lei et al., 1991; Young et al., 1993; Cromwell et al., 1994; Power and Kahn, 1993). These studies showed one or more of the following improvements: increased overall phosphorus digestibility, improved phytate phosphorus availability, increased growth rate, increased feed efficiency, improved ileal protein digestibility and protein deposition, increased bone strength and decreased fecal phosphorus.

Nelson et al. (1968) were the first to report an improvement in the availability of phytate phosphorus in chicks due to supplemental phytase. The enzyme, produced by a culture of *Aspergillus ficuum* (strain NRRL 3135), was added to liquid soybean meal and incubated at 50^0C for 24 hours. When treated dried

soybean meal was fed to 1-day old chicks, a considerable increase in bone ash was observed compared to controls receiving no inorganic phosphorus. Thereafter, Nelson et al. (1971) demonstrated that supplemental phytase added directly to chick diets increased in vivo utilization of phytate phosphorus. The addition of phytase produced by *Aspergillus ficuum* to diets as a dry powder produced an increase in percentage bone ash and increased rate of gain in White Leghorn cockerels. Total hydrolysis of phytate was achieved when 3 g phytase supplement was used per kg diet. Chicks utilized phosphorus from phytate as well as supplemental phosphate from sodium orthophosphate or β-tricalcium phosphate. Simons et al. (1990) confirmed findings of Nelson et al. (1968, 1971) in a series of experiments with broiler chicks fed diets based on maize and grain sorghum supplemented with soybean and sunflower meals. Phytase tested by these authors was produced by the same strain of *Aspergillus ficuum* that was used by Nelson et al. (1968, 1971). The apparent *in vivo* availability of phosphorus was improved by adding different levels of microbial phytase. Growth rate and feed conversion ratio of broilers were dependent on levels of supplemental phytase. Additionally, mortality was decreased in birds fed treated diets.

STATEMENT OF THE PROBLEM

A great deal of research has been conducted with supplemental enzymes in barley-, oat-, and rye-based diets, whereas only limited data is available on the use of enzymes in wheat-based diets. β-Glucans found in oats and barley, and arabinoxylans of oats, barley, rye and wheat are known to be anti-nutritional factors in these grains. These cause an increase in viscosity of digesta thus restricting nutrient absorption from the gastrointestinal tract. This effect alters absorption of basic nutrients, such as fat and protein, and the utilization of calcium, phosphorus and zinc. Supplemental β-glucanase and pentosanases have been reported to facilitate the digestion of β-glucans and pentosans in barley-, wheat-, and rye-based poultry diets resulting in improved bird performance, improved litter quality, reduced nitrogen and phosphorus in fecal waste, and reduced mortality (Hesselman et al., 1982; Petterson and Åman, 1988).

Phytic acid, (myoinositol 1,2,3,4,5,6-hexakisphosphate), is ubiquitously distributed throughout the plant kingdom and is found in the blood of amphibians, reptiles, and birds. In plants, phytic acid (phytate) serves as a reservoir for phosphorus and acts as a metabolic ballast ensuring seed dormancy. Phytate is the major phosphorus-containing compound in cereals, comprising approximately 70% of the total phosphorus. Because of its highly ionized orthophosphate groups, it readily complexes with a variety of divalent cations and proteins in the gastrointestinal tract of animals. It is this trait which categorizes phytate as an anti-nutritional factor because it decreases the bioavailability of proteins and nutritionally

important minerals such as calcium, zinc, magnesium and iron. In addition to its anti-nutrient activity, phytate phosphorus is unavailable for use by monogastrics due to the absence of sufficient levels of endogenous phytase in their digestive tracts (Power and Kahn, 1993).

Hypothesis and Objectives

Hypothesis

The presence of arabinoxylan in the cell wall structure of wheat aleurone and endosperm tissue possibly inhibits the digestibility of starch and protein by preventing contact with digestive enzymes. Fiber degrading enzymes, such as xylanase may enhance the digestive process by permitting greater enzyme/substrate contact.

The location of phytate in the aleurone layer of wheat kernels, suggests that the use of fiber-degrading enzymes such as β-glucanase and pentosanase in combination with phytase, could possibly enhance the activity of the latter or endogenous phytases in the gastrointestinal tracts of monogastric animals.

Objectives

(1) To evaluate the effectiveness of a mixture of supplemental xylanase, protease, and phosphatase on the production performance of broiler chickens fed wheat-soybean meal diets, and

(2) To evaluate the ability of these enzymes to release phytate from the plant cell walls, exposing the compound to endogenous microbial phytases present in the gastrointestinal tract of chickens.

MATERIALS AND METHODS

Grains, Preparation, Sampling, Tissue Collection and Chemical Analyses

Three types of wheat were used in this study, hard red spring (HRS), hard red winter (HRW) and soft white (SW). All were grown near Bozeman MT in 1991. The HRS and SW wheats were provided by Western Plant Breeders, Inc., Bozeman MT and the HRW wheat was grown on the Montana Agricultural Experiment Station farm. The three wheats were compared in Experiment 1 with and without enzyme. HRW wheat was fed in Experiment 2 and 3 to test the effect of level of phosphorus and enzyme mixture. Experiments 1 and 3 were conducted for three weeks and Experiment 2 for six weeks. Corn was fed instead of wheat in adaptation diets. Soybean meal (44% protein), obtained from a local feed supplier, was used for supplemental protein in all diets. Prior to incorporation into diets, the corn, wheats and soybean meal were ground through a 3.175 mm hammer mill screen. For analyses, representative samples of the ground grains and soybean meal were further ground in a Udy cyclone sample mill through a .5 mm screen. Analyses of the grains and soybean meal included dry matter, protein, ether extract (Anonymous, 1971), acid detergent fiber, ash, (AOAC, 1980), calcium (Clark and Collip, 1925), phosphorus (Fiske and SubbaRow, 1925), phytic acid (HRW wheat; Raboy et al., 1984), total dietary fiber, insoluble dietary fiber, and soluble dietary

fiber (Lee et al. 1992). Relative extract viscosity was determined on the wheats as described by Aastrup (1979).

In Experiments 1 and 3, total fecal collections were performed on days 11, 12, 13, and 18, 19, and 20. In Experiment 2, fecal waste was collected on days 11, 12, 13 and 32, 33 and 34. Representative samples of the fecal material were freeze-dried, stored at -20^{0}C for later analyses of dry matter, protein (AOAC, 1980), ether extract (Anonymous, 1971), calcium (Clark and Collip, 1925), and phosphorus (Fiske and SubbaRow, 1925). In Experiments 2 and 3, birds were killed by CO2 asphyxiation. Wing bones (radius and ulna) were taken and analyzed for ash (AOAC, 1980). Prior to analyses, wing bones were cleaned of all soft tissue, freeze-dried and defatted with acetone.

Chicks and Experimental Conditions

One-day-old Hubbard cockerel broiler chicks, obtained from Fors Farms, Puyallup WA, were housed in groups in battery type cages with wire mesh floors. Chicks were fed a standard 23% protein corn-soybean meal diet (Table I) for three days before the start of each experiment. During this period the birds were wing banded for identification. Birds were housed in a room in the MSU Animal Resource Center with continuous lighting, maintained at 29.0^{0}C for three weeks and 24.0^0 C from three to six weeks where birds were fed to the latter age in experiment 2. Cage temperature was maintained by thermostatically controlled heaters; temperatures were reduced from start to three weeks from 35^{0}C to 26.7^{0}C. Cages were not heated from three to six weeks. Diets and water were provided *ad libitum*

for each treatment. Experiment 1 contained four replicates of eight birds per group for a total of 192 birds. Experiment 2 contained three replicates of 10 birds per group for a total of 240 birds. Experiment 3 contained three replicates of eight birds per group for a total of 192 birds. Individual body weights were recorded initially, weekly, and at the conclusion of each experiment. Feed consumption for each cage was recorded at the end of each week and at the end of the experiment. Birds that died or were removed due to deformed legs during the course of the study were identified, weighed and the date and cage number recorded. Feed consumed by chicks that died before completion of experiment was taken into account while calculating the total feed intake of each cage group. Feed/gain (F/G) ratio was calculated weekly for each group and for the overall feeding period.

Experiments and Diets

A total of 192 male Hubbard broiler chicks were used in Experiment 1 to determine the influence of wheat type (hard red spring (HRS), hard red winter (HRW), soft white (SW) with and without dietary supplemental enzymes (Avizyme®, Finnfeeds International, Marlborough UK) on average daily gain, average daily feed intake, feed efficiency, fecal composition, and bone ash. Avizyme is an enzyme mixture containing xylanase, amylase and protease enzymes. Six wheat-soybean meal based rations were formulated to contain 21% protein and 1.2% lysine (NRC, 1984) (Table 1). NRC, (1984) recommended minimum levels of all nutrients were met or exceeded in all diets. Cornstarch was added in the nonsupplemented diets to compensate for the enzyme added in the supplemented diets. This experiment

consisted of chicks in four replicate groups of six dietary treatments in a 3x2 factorial treatment arrangement.

In the second experiment a total of 240 male Hubbard day old broiler chicks were used to determine the influence of four levels of total dietary phosphorus (0.54%, 0.49%, 0.44%, and 0.39%) on a HRW wheat-soybean based diet with and without enzyme xylanase (Table 2) on average daily gain, average daily feed intake, feed efficiency, fecal composition, and bone ash. After three weeks of feeding, one-half of the birds from each cage were sacrificed for bone analysis and the remainder killed and bones taken for analysis at the end of six weeks. This experiment consisted of 10 chicks in three replicate groups of eight dietary treatments in a 4x2 factorial arrangement of treatments.

In Experiment 3, a total of 192 day old male Hubbard broiler chicks were used to determine the influence of six levels of an enzyme mixture containing xylanase, protease and phosphatase enzymes on averages daily gain, averages daily feed intake, feed efficiency, fecal composition, and bone ash. This experiment consisted of eight chicks in three replicate groups of eight dietary treatments. Seven diets were formulated to contain 0.55% total phosphorus with the following enzyme supplements: Diet 1, negative control with no enzyme; diet 2, 0.0002 % phosphatase; diet 3, 0.001% phosphatase; diet 4, 0.005% phosphatase; diets 5, 6 and 7 were prepared as diets 2, 3, and 4 + 0.1% xylanase/protease mixture + 0.02% xylanase, respectively. An eighth diet was formulated to contain 0.67% total phosphorus with no enzyme supplement for a positive control (Table 3).

Statistical Analysis

Data were analyzed by ANOVA using the General Linear Model procedure (SAS, 1985). The interaction between wheat type and enzyme supplement was tested in Experiment 1. In Experiment 2, the data were analyzed for linear, quadratic and cubic effects. Differences between means were compared by Least-Square Means using MSUSTAT (Lund, 1987).