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ABSTRACT OF DISSERTATION

Tuoying Ao

The Graduate School
University of Kentucky
2005

EXOGENOUS ENZYMES AND ORGANIC ACIDS IN THE NUTRITION OF BROILER CHICKS: EFFECTS ON GROWTH PERFORMANCE AND IN VITRO AND IN VIVO DIGESTION

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agricultural at the University of Kentucky

By

Tuoying Ao

Lexington, Kentucky

Director: Dr. Austin H. Cantor, Associate Professor of Animal Sciences

Lexington, Kentucky

2005

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ABSTRACT OF DISSERTATION

EXOGENOUS ENZYMES AND ORGANIC ACIDS IN THE NUTRITION OF BROILER CHICKS: EFFECTS ON GROWTH PERFORMANCE AND IN VITRO AND IN VIVO DIGESTION

Studies were conducted to investigate the interactive effects of exogenous enzymes and organic acids on *in vitro* and *in vivo* nutrient digestion and growth performance of broiler chicks. In Study 1, five exogenous enzyme products including β-glucanase, xylanase, amylase, α-galactosidase and protease, were assayed in triplicate at their optimum pH levels and at pH levels of 3.0, 6.0, 6.5, 7.0 and 7.5, which were used to simulate pH levels found in the gizzard, the diet, the crop, and the proximal and distal parts of small intestine, respectively. The pH gradient was obtained by dissolving the enzymes in different buffers. Results suggested that the pH levels commonly found in the avian digestive tract were either too high or too low for maximum activity of the exogenous enzymes, such as α-galactosidase and protease.

In Study 2, broiler chicks were fed corn basal, barley basal or wheat basal diets with different levels or different sources of organic acids. Dietary inclusion of graded levels of organic acids linearly reduced the pH of the diet and crop content, but not the pH of the digesta sampled in the gizzard and small intestine. The inclusion of 2% organic acids (citric acid or fumaric acid) in broiler diets had either no effect or negative effects on chick growth performance.

In Study 3, an *in vitro* model was used to simulate the chicken's digestive process in the crop, the gizzard and the small intestine. Soybean meal and raw whole soybean

were used as substrates. Graded levels of either α-galactosidase (0 to 13,792 units/kg) or protease (0 to 888 units/kg) and 0 or 2% citric acid were added to the substrates in a factorial arrangement. Reducing sugars, α-amino nitrogen and trypsin inhibitor content were measured. The data indicated that increasing levels of α-galactosidase linearly increased the release of the reducing sugars from the soybean meal. Addition of citric acid further increased the activity of α-galactosidase, resulting in more reducing sugars were released. Increasing the supplementary levels of protease linearly increased the α-amino nitrogen release from the soybean meal and raw whole soybean. Trypsin inhibitor content in the raw whole soybean was not influenced by the application of the protease.

In Study 4, broilers were fed low energy or normal energy basal diets with α-galactosidase, amylase and acidification of diet and water. Growth performance, AME_n and digestibility of DM, CP and NDF were observed. Alpha-galactosidase improved the AME_n of the diets and increased the weight gain and feed intake of broiler chicks. Citric acid decreased the crop pH and enhanced the activity of α-galactosidase in the crop. Citric acid decreased the AME_n of the diets and chick growth performance. These effects were corrected by supplementing α-galactosidase.

The activity of α-galactosidase was enhanced by simultaneously using organic acid. The negative effects on chick growth performance by dietary inclusion of organic acids were corrected by simultaneously using α-galactosidase.

KEYWORDS: exogenous enzymes, organic acids, broilers, digestibility, growth performance

Tuoying Ao

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DISSERTATION

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CHAPTER 1: INTRODUCTION

Various supplements of exogenous enzymes have been used in poultry diets to improve feed utilization. These enzymes include β-glucanase, xylanase, amylase, α-galactosidase, protease, lipase and phytase. The exogenous enzymes are used either to correct the lack of specific endogenous enzymes for digesting certain nutrients in various feedstuffs or to hydrolyze anti-nutritional factors in feed ingredients (Annison, 1993; Bedford and Schulze, 1998; Simon, 1998; Sheppy, 2001).

When the enzymes are added to diets, they must be active under the physiological conditions prevailing in the animal's digestive tract in order to realize their functions. Also, enzyme activity in poultry diets must be sufficiently high to compensate for the relatively short transit time. Enzyme activity is markedly influenced by the pH of the digesta. Studies comparing the efficacy of the carbohydrase enzymes between pigs and chicks indicated that the low pH in the stomach of the pig was detrimental to most of exogenous enzymes (Baas and Thacker, 1996; Thacker and Baas, 1996). In poultry, the presence of the crop may have contributed to the higher efficacy of the exogenous enzymes (Chesson, 1993; Dierick and Decuypere, 1996; Bedford and Schulze, 1998; Danicke *et al.,* 1999; Partridge, 2001). Therefore, the crop of the chicks may play a very important role in affecting the efficacy of the exogenous enzymes supplemented to the diet. Almost all of the exogenous enzyme products used in animal diets is either from fungal or from bacterial sources. Studies showed that both fungal and bacterial enzyme preparations have optimum activity at pH 4.0-5.0 (Shieh *et al.*, 1969; Coughlan, 1985; McClear and Glennie-Holmes, 1971; Grootwassink *et al.*, 1989; Simons *et al.*, 1990; Baas and Thacher, 1996; Ademark *et al.*, 2001). However, during the initial residency of the diet in the crop (about 2.5 - 3.0 h), the average pH level is about 6.5 (Bolton, 1965; Herpol and Van Grembergen, 1967; Riley and Austic, 1984). Studies by Bass and Thacker (1996) demonstrated that the enzyme activities of both β-glucanase and pentosanase at pH 6.5 were only 10~15% of the activities at pH 4.5 or 5.5.

Organic acids have been used for decades in protecting feed from microbial and fungal destruction. It is well documented that organic acid supplementation can reduce the pH of the diet and stomach digesta in pigs (Kirchgessner and Roth, 1982b; Falkowski and Aherne, 1984; Giesting and Easter, 1985; Radcliff *et al.*, 1998). Acidification of the diet can promote growth performance of piglets and broilers and increase the digestibility of crude protein and amino

acids (Vogt *et al.,* 1979, 1981; Giesting and Easter, 1985; Gabert and Saucer, 1994; Partanen and Mroz, 1999). Dietary addition of organic acids can also improve the digestibility of minerals and increase the utilization of the phytate phosphorus (Shohl 1937; Pileggi *et al.* 1956; Boling *et al.*, 1999, 2000; Li *et al.,* 1998; Edwards and Baker, 1999). In poultry production, organic acids have been widely used to inhibit pathogens like *Salmonella spp.* in both raw material and finished feed and to sanitize the drinking water (van der wal, 1979; Radcliffe, 2000; Broek *et al.*, 2003). Organic acids and exogenous enzymes have also been considered as substitutes of growth promoters in recent years (Verstegen and Williams, 2002).

Most poultry diets in the United States are based on corn and soybean meal. These ingredients are generally considered to be highly digestible and nutritive to the bird. However, the metabolizable energy (ME) of soybean meal is quite low when compared with its gross energy (Pierson *et al.*, 1980; Coon *et al.*, 1990). This is due mainly to the very poor digestibility of the carbohydrate fraction. Soybean meal contains up to 22.7% non-starch polysaccharides (NSPs) on a dry matter basis (Chesson, 1987). This includes about 6% oligosaccharides including 1.0% raffinose and 4.6% stachyose (Trugo *et al.*, 1995). These oligosaccharides cannot be digested in the small intestine of poultry because of the absence of endogenous α-(1,6)-galactosidase enzyme (Gitzelman and Auricchio, 1965). Undigested oligosaccharides can negatively influence growth rates and protein digestion of young chicks and pigs (Veldman *et al.,* 1993; Gdala *et al.,* 1997) and cause flatulence in rats, dogs, and human being (Steggerda, 1968; Rachis *et al.,* 1970). There is evidence that the ME of soybean meal can be improved through using exogenous α-galactosidase (Graham *et al.*, 2002).

These trials were conducted to test the hypothesis that supplementing poultry diets containing the exogenous enzymes with organic acids will enhance the enzyme efficacy through optimizing the pH of the crop content.

CHAPTER 2: LITERATURE REVIEW

The Use of Enzymes in Poultry Rations

Enzymes are defined as protein catalysts that initiate and control the rate of biological reactions that change substrates into products. Throughout history, people have made use of enzyme-catalyzed reactions for such processes as brewing, baking, and antibiotic synthesis. Since the first enzyme - urease was isolated and purified by Sumner in 1926, isolated enzymes have been widely used in the textile, leather, food and beverage industries. Enzymes have been used in animal feed for more than 50 years, but the rapid growth in their use has only been within the last 10 years. The most common application of exogenous enzymes is in the feeding of the broiler chickens (Sheppy, 2001).

Enzymes play a key role in the digestive process. Although enzymes are produced by the animal itself or by the microbes naturally present in the digestive tract, specific activities necessary to break down some compounds in feed are not found or are at low levels in the digestive tract. Therefore, exogenous enzymes are added to the diet to break down these compounds. Many years ago, nutritionists had generally regarded enzyme addition to diets as a futile effort on the basis that proteolysis in the stomach and anterior small intestine would result in inactivation before they could be of significant digestive benefit. However, in 1946, Hastings first reported that addition of a diastatic enzyme material to a high fiber chick diet improved growth and feed efficiency. Later, Jensen *et al.* (1957) found that supplementation of barley-based poultry diets with a crude mixture containing β-glucanase activity gave a significant improvement in the performance of the birds as well as an improvement in litter quality. Nelson *et al.* (1967) also pointed out the effectiveness of a microbially produced phytase for increasing the utilization of phosphorus from plant sources by poultry. Since then, a lot of research work has been done concerning these two enzymes and other feed enzymes.

Feed enzymes are mainly produced from microorganisms by a process of fermentation and extraction. The enzyme products often do not contain a single activity. The benefits of enzymes in feed applications include: 1) digesting substrates that cannot be hydrolyzed by endogenous enzymes; 2) elimination of anti-nutritional factors; and 3) increasing utilization of the feed (Annison, 1993; Bedford and Schulze, 1998; Simon, 1998; Dudley-Cash, 2001; Sheppy, 2001). As a result of the advances in biotechnology over the past 10-20 years, especially in the areas of

genetics and protein engineering, there is now widespread use of enzymes in poultry rations. The types of feed enzymes currently available are outlined in Table 2.1.

Table 2.1. Enzymes used in poultry rations today

Enzyme	Substrates	Target feedstuff	Expected benefits
β-Glucanase	β-Glucan	Barley, oats and rye based diets	Reduction of sticky droppings, improved feed utilization
Amylase	Starch	High starch cereal diets	Increased availability of cereals
Xylanase	Arabinoxylan	Rye, barley and wheat	Improved litter quality, improved feed utilization
α-Galactosidase	Oligosaccharides	Soybean and other legumes	Improved energy availability
Phytase	Phytate	Many different diets	Reduces need for inorganic phosphorus
Protease	Protein and ANFs	wheat by-products, legume proteins	Higher protein digestibility, lower nitrogen excretion
Lipase	Fat	Animal and vegetable fats	Improved digestibility of fat and enhanced energy retention as a result

From Thorpe and Beal, 2001

Non-Starch Polysaccharides (NSPs) and Carbohydrate-Degrading Enzymes

The most important enzymes in the poultry feed industry today are β-glucanase, xylanase and phytase. Both β-glucanase and xylanase are carbohydrate-degrading enzymes. They are widely used in poultry diets containing barley, oats, rye or wheat to improve feed utilization by removal of the anti-nutritional effects of NSPs contained in these cereal raw materials. The NSPs are the major components of the cell wall of cereals. Some of them located mainly in endosperm cell walls of cereals are soluble and others are insoluble. Originally, NSPs were thought to be inert or make minor contributions to the nutrition of chickens. However, research in recent decades showed that soluble NSPs possess anti-nutritive activity. In the case of barley and oats, these soluble polysaccharides are mainly mixed linked β-glucans and in rye and wheat they are

mainly arabinoxylans. Typical values for the content of these NSPs in several grains are shown in Table 2.2.

Table 2.2. The total and water-soluble arabinoxylan and β-glucan content of cereal grains (g/kg) and the percentage contribution of the arabinoxylan and β-glucan of starchy endosperm walls to the grain total

Cereal	Arabinoxylan			β-Glucan		
	Grain total	Grain soluble	Endosperm (%)	Grain total	Grain soluble	Endosperm (%)
Barley	56.9	4.8	22	43.6	28.9	99
Oats	76.5	5.0	12	33.7	21.3	47
Rye	84.9	26.0	44	18.9	6.8	71
Wheat	66.3	11.8	35	6.5	5.2	48

(From ESC, 2004)

Fry *et al.* (1957) reported that a high level of barley in poultry diets caused depressed growth and poor feed efficiency, coupled with sticky droppings, especially in young chicks. Many studies (Willingham *et al.,* 1959; Anderson *et al.,* 1961; Adams and Naber, 1969; Gohl *et al.,* 1978) showed that water-treatment of barley increased the nutritive value to a level equal to that of corn. White *et al.* (1981) confirmed that the major anti-nutritional factor in barley was β-glucans.

Inclusion of rye in poultry diets has been fraught with problems, principally related to the production of sticky droppings and particularly poor growth and feed conversion in younger chicks (Moran *et al.,* 1969). MacAuliffe *et al.* (1976) found that rye contained a water extractable factor that depressed growth and produced sticky and watery excreta in chicks. Misir and Marquardt (1978) hypothesized that the anti-nutritive factor in rye was probably due to a polysaccharide present in rye grain. Antonion *et al.* (1981) isolated pentosans from rye and incorporated them into experimental diets. Significant depressions occurred in chick growth, feed utilization and the digestibilities of fat and amino acids. Fengler and Marquart (1988) confirmed that the rye anti-nutritional compounds are pentosans.

Inclusion of whole wheat at a high level in broiler diets also had negative effects on performance (Rogel *et al.*, 1987). Choct and Annison (1990) demonstrated the anti-nutritional effect of pentosans in wheat.

Soluble NSPs in cereals such as β-glucans and pentosans cannot be digested and utilized by monogastric animals, such as chicks, due to the absence of necessary enzymes in the gastrointestinal tract (White *et al.*, 1983). These NSPs are soluble in water producing viscous solutions (Figure 2.1). Increased viscosity of the fluid film surrounding the villi of rat jejunum gave rise to a thickening of the rate-limiting unstirred layer (Johnson and Jennifer, 1981). This caused accumulation of nutrients and a reduction of nutrient absorption *in vitro* (White *et al.*, 1983). The inclusion of barley, rye and wheat in poultry diets significantly increased the viscosity of the digesta in the small intestine, resulting in a decreased rate of diffusion and interaction of substrates and digestive enzymes at the mucosal surface (White *et al.*, 1983; Hesselman and Aman, 1986; Edwards *et al.,* 1988; Ikegami *et al.,* 1990). This not only affects the absorption of basic nutrients such as glucose, fatty acids and amino acids (Fengler and Marguardt, 1988), but also affects the utilization of certain minerals, such as calcium, phosphorus and zinc (Gordon, 1990). Studies by Low (1989) demonstrated that the endogenous secretion of water, proteins, electrolytes and lipids can be increased markedly by adding NSPs to the diet.

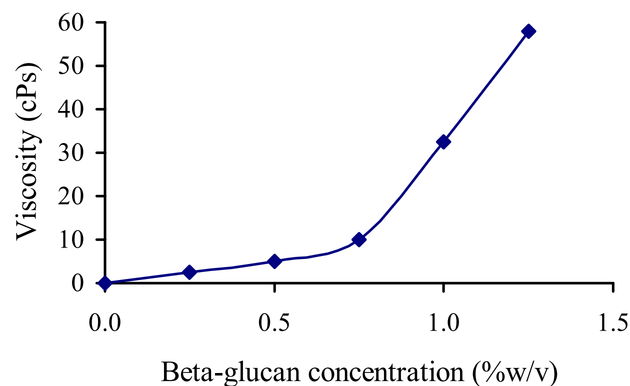

Figure 2.1. The viscosity of β-glucan solutions (from ESC, 2004)

MacAuliffe and McGinnis (1971) showed that the negative effect of feeding a high level of rye in poultry diets can be largely overcome by addition of antibiotics. They suggested that the higher content of pentose sugars in rye may be important in stimulating the growth of harmful microorganisms in the intestinal tract. Choct at al. (1996) demonstrated a negative influence to the gut microorganism of birds by increasing amount of NSPs in the digesta. Increased microbial load in the small intestine can increase the turnover of intestinal cells due to some of their fermentation products (Lesher *et al.,* 1964; Osborne and Seidel, 1989).

Jensen *et al.* (1957) reported that enzyme supplementation significantly improved both growth rate and feed efficiency in chickens fed pearled barley-based control diets. Later, in 1962, Ricks *et al.* found the enzyme-induced improvement in the feeding value of barley is from the endo-β-glucanase activity. Numerous studies confirmed this finding (Fry *et al.,* 1958; Willinghum *et al.*, 1959; Berg, 1961; Potter *et al.*, 1965; Hesselman *et al.*, 1981; Hesselman and Aman, 1986; Cantor *et al.*, 1989; Brenes *et al.*, 1993). Many studies also demonstrated that the performance of rye-fed or wheat-fed birds can be improved by dietary supplementation with enzyme products containing xylanase activity (Pettersson and Aman, 1988; Grootwassink *et al.*, 1989; Classen and Campbell, 1990; Friesen *et al.*, 1991; Bedford *et al.,* 1991).

The first impression of many researchers is that the improvement in performance in relation to enzyme supplementation is due to complete hydrolysis of the polysaccharides and subsequent absorption of the released sugars. However, the assay conducted by White *et al.* (1983) showed that the growth improvement of chicks fed enzyme-supplemented barley diets could not be due to improved glucose availability from β-glucans. Baker (1977) investigated the utilization of xylose and xylan by the chick. He pointed out that xylan yielded no useful energy to the chick and a high level of xylose in the diet appeared to be toxic. This means that even if the xylans were completely hydrolyzed by the enzymes, the released monosaccharides may not be efficiently utilized. So far, it is generally conceded that the reduction in viscosity achieved by endolytic enzyme activity is responsible for the majority of the improvement in performance seen in young chicks (Campbell *et al.,* 1986; Chesson, 1987; Rotter *et al.,* 1989b; Classen and Bedford, 1991; Cowan, 1992; Bedford and Schulze, 1998).

The Use of Enzymes in Corn-Soybean Meal-Based Poultry Rations

Corn is an excellent energy source and the major cereal grain fed to poultry and swine in the world. Soybeans accounted for 56% of the world oilseed production (American Soybean

Association, 2003). The animal feed industry uses 77% of the soybean meal produced primarily as an amino acid and protein source in diets (Kerley and Allee, 2003). In regions such as the United States and Asia, poultry feed is based primarily on corn and soybean meal. The nutrients contained in corn and soybean meal are generally considered to be highly digestible. Heat treatment is commonly used to inactivate antinutritive factors (ANFs) such as protease inhibitors and lectins in soybean meal (Campbell and van der poel, 1998). However, the energy utilization in corn and soybean meal also depends on the amount of indigestible carbohydrates present, particularly oligosaccharides. The inclusion of high concentrations of soybean meal as the sole protein source in broiler diets can adversely affect growth performance (Irish and Balnave, 1993). In addition, some of ANFs in soybean, such as antigenic proteins and phytate cannot be reduced or alleviated by heat. With the recent developments in feed enzyme technologies, many microbial enzymes such as phytase, amylase, protease and α-galactosidase, have been used into corn-soybean meal-based diet either to improve digestibility of nutrients or to reduce the ANFs.

Most of the stored phosphorus in plants is found in seeds, mainly as phytate phosphorus. In corn, 90% of the phosphorus is present as phytate, and in soybean meal 75% of the phosphorus is present as phytate (CVB, 1998). Phytate phosphorus is poorly available (30%) to monogastric animals, including poultry, due to the absence of adequate levels of endogenous enzyme phytase or phosphatase. Actually, phytate is often considered toxic, or antinutritive (Pallauf and Rimbach, 1997), because it is capable of binding di- and trivalent cations such as Ca, Co, Cu, Fe, Mg, Mn, Ni and Zn in very stable complexes (Wise, 1983) and reducing the availability of these minerals to the animal (Pallauf and Rimbach, 1997). In addition, phytate may form complexes with proteins and starches and may also reduce the availability of these nutrients from the diet (Graf, 1986; Thompson, 1986). Dietary supplementation with microbial phytase is well established as an effective and practical method of improving phytate digestibility in production animals (Kornegay, 2001). In poultry, microbial phytase supplementation generally results in a 20-45% improvement in phytate-P utilization (Ravindran *et al.*, 1995). The negative effect of phytate on mineral digestibility is ameliorated by dietary supplementation with microbial phytase. Microbial phytase supplementation of corn-soybean meal-based diets improved Ca availability and Zn utilization in poultry (Sebastian *et al.,* 1996a,b) and increased the apparent absorption of Mg, Zn, Cu and Fe in pigs (Adeola, 1995). Phytase supplementation also increased

the ileal digestibility of crude protein, and most amino acids in both poultry and swine (Sebastian *et al.,* 1997; Yi *et al.,* 1996; Mroz *et al.,* 1994).

The energy utilization of soybean meal by poultry is very poor. The digestibility of the dry matter and gross energy in soybean meal is approximately 50% when fed to poultry (Dudley-Cash, 2001). The metabolizable energy (ME) value of dehulled soybean meal suggested by the National Research Council (NRC) bulletin for swine (1998), is 3,380 kcal/kg. The NRC bulletin for poultry (1994) suggests a ME value of only 2,240 kcal/kg for dehulled soybean meal when fed to poultry. Pierson *et al.* (1980) pointed out that the low ME of soybean meal for poultry is due mainly to the very poor digestibility of the carbohydrate fraction. Soybean meal contains up to 22.7% NSPs on a dry matter basis (Chesson, 1987). This includes about 6% oligosaccharides, including 1.0% raffinose and 4.6% stachyose (Trugo *et al.*, 1995). These oligosaccharides cannot be digested in the small intestine of poultry because of the absence of endogenous α-(1,6)-galactosidase enzyme (Gitzelman and Auricchio, 1965). In addition to their indigestibility, these oligosaccharides have been shown to produce gastrointestinal gas in rats, dogs, and man (Steggerda, 1968) and produce diarrhea that may increase digesta passage rate and decrease digestion and absorption of nutrients (Kuriyama and Mendel, 1917; Wiggins, 1984). Coon *et al.* (1990) studied the effect of oligosaccharide-free soybean meal on the ME content of soybean meal and fiber digestion in adult roosters. The results showed that the removal of the oligosaccharides in soybean meal by ethanol extraction increased the nitrogen corrected true metabolizable energy (TME_n) by 21% due to increased fiber digestion and the digesta passage rate was reduced by approximately 50%. Further studies by Coon and coworkers (Leske *et al.*, 1991, 1993) also demonstrated the improved TME_n of soybean meal through alcohol extraction with both roosters and broilers. The recombination of the alcohol extract or addition of pure raffinose and stachyose to soy protein concentrate yielded TME_n values that were similar to those of soybean meal. Parsons *et al.* (2000) compared the AME_n of soybean meals varying in oligosaccharide content using roosters. The results indicated that the TME_n of low oligosaccharide soybean meal was significantly higher than that of conventional soybean meals. However, Irish *et al.* (1995) removed up to 90% oligosaccharides from soybean meal using either ethanol extraction or exogenous α-galactosidase. No beneficial effect on the nutritional value of soybean meal was observed when the low oligosaccharide (extracted or enzyme-incubated) soybean meal was fed to broiler chicks.

Many studies have been conducted to investigate the effect of carbohydrase and protease supplementation to corn-soy diets on the nutritive value of diets and performance of chicks. Swift *et al.* (1996) examined the effects of a commercial enzyme product called Allzyme Vegpro, a mixture of protease, cellulase, pentosanase, α-galactosidase and amylase, on digestibility and growth performance of broiler chicks. Enzyme treatment significantly improved nitrogen and energy digestibility and feed conversion over a 35-day feeding period. Schang *et al.* (1997) compared Vegpro in corn-soybean meal and corn full-fat soybean diets for broilers, using high and low nutrient density formulations. Addition of the enzyme product to the low density diet significantly improved body weight gain and feed conversion. Marsman *et al.* (1997) examined the effect of enzyme treatments (protease and carbohydrase) of soybean meal on growth performance and ileal nutrient digestibilities in broiler chicks. Enzyme treatment improved apparent ileal digestibility of crude protein and NSPs; however, enzyme treatment did not improve growth performance of the chicks. Zanella *et al.* (1999) investigated the effect of a commercial enzyme cocktail containing xylanase, protease and amylase on performance of broilers fed a corn-soybean meal based diet. Enzyme supplementation improved body weight gain, feed conversion ratio and ileal digestibility of crude protein. Graham *et al.* (2002) pretreated soybean meal using 4% α-galactosidase enzyme solution. Enzyme treatment degraded raffinose and stachyose in soybean meal by 69 and 54%, respectively, compared to untreated soybean meal. Enzyme treatment increased TME from 2974 to 3328 kcal/kg. However, chick growth performance was not significantly improved by enzyme treatment. Kocher *et al.* (2002) investigated the effect of two enzyme products on the nutritive value of soybean meal with emphasis on changes in composition of NSPs along the digestive tract. They concluded that glycanases with galactanase and pectinase activities supplemented at appropriate dosages can improve the digestibility of the NSPs in soybean meal and increase the metabolizable energy content of the diet containing high levels of soybean meal. In another report, Kocher *et al.* (2003) pointed out that although enzyme addition to the corn-soybean meal based diet can significantly improve AME_n, the improvement depended greatly on the raw ingredients available at the time. Studies by Ghazi *et al.* (2003) demonstrated the improvement of the nutritive value of soybean meal by protease and α-galactosidase treatment in broiler chicks. They first used tube-fed chicks to measure the effect of different enzyme treatments on true metabolizable energy (TME) and true nitrogen digestibility (TND) of commercial solvent-extracted, heat-treated soybean meal.

Protease and α-galactosidase improved TME and TND of the soybean meal. In other studies, they added enzymes in broiler diets and fed broilers for 21 d. Increases in chick growth rate and digestibility that were similar to those recorded in previous study were obtained when protease and α-galactosidase were included in the diets.

Parkany-Gyarfas (1975) found a 3.6% improvement in body weight and 4.0% improvement in feed utilization in male turkeys when a corn-soybean meal diet was supplemented with α-amylase. Similar results were observed by Ritz *et al* (1995). The later study also demonstrated that the mean villus length within the jejunum and ileum was significantly increased at 2 and 3 wk of age by dietary supplementation of amylase when compared with control diet. These findings suggest that the increased growth associated with the amylase diet be explained in part by the increase in absorptive surface area, allowing for increased digestion of available nutrients coupled with increased enzyme activity. However, no physiological mechanism to explain increased villus length as a response to enzyme supplementation is known. In chicks, Noy and Skilan (1995) reported that daily net secretion of amylase was low at d 4 and steadily increased up to d 21. Uni *et al.* (1995) also reported that the secretion of amylase per gram of feed was low at d 4, increased up to d 7, and then stabilized. Burnett (1966) first reported the beneficial effects of amylase and protease preparations on growth and feed efficiency of chicks when added to diets. Gracia *et al.* (2003) studied the influence of exogenous α-amylase on digestion and performance of broilers fed a corn-soybean meal diet. At 7 d age, α-amylase supplementation improved daily gain by 9.4% and feed conversion by 4.2%. Also, α-amylase supplementation significantly improved apparent fecal digestibility of organic matter and starch and AMEn of the diet. The weight of pancreas as a percentage of body weight decreased with α-amylase supplementation, which indicates that the secretion of pancreatic enzymes might be affected by the concentration of enzymes and substrates or products of their hydrolysis in the lumen of the small intestine.

Scheideler *et al.* (1999, 2003a, b) conducted several studies to investigate the effect of enzyme addition to corn-soybean rations on pullet and laying hen performance. They used a commercial enzyme product called Avizyme 1500 that is a microbial multi-enzyme package with amylase, protease and xylanase activity. The results showed that the enzyme supplementation increased pullet growth rate and improved egg production, egg mass and feed conversion ratio. Improvements were also seen in nitrogen retention and availability of energy in pullet and layer

diets supplemented with enzyme. In another study, Scheideler and Weber (2003) investigated the role of α-galactosidase in corn-soy based layer rations. They found the addition of α-galactosidase improved egg production of hens and the ME of the diet. Hens performed very well on diet formulations reducing the energy available from fat sources and relying on more energy from soybean meal when α-galactosidase was added to the rations. Gomez *et al.* (1999) added Avizyme 1500 to corn-soybean meal diets with three energy densities. Enzyme supplementation improved egg mass and feed conversion ratio at all three energy levels tested.

Pretreatment of raw soybean or soybean meal with proteases was studied in many experiments. The purpose of adding proteases in soybean or soybean meal containing diet is to destroy or inactivate the anti-nutritional factors, such as residual trypsin inhibitors, lectins and antigenic protein. Huo *et al.* (1993) found that fungal and bacterial protease enzymes could inactivate trypsin inhibitors and lectin in raw soybean and low-temperature extruded soybean *in vitro*. Based on their results, the protease from bacterial source was more effective at breaking down trypsin inhibitors than the protease from fungal source. Rooke *et al.* (1996) incubated the soybean meal using 0.1% acid protease for 3 h at 50˚C, pH 4.5. The soybean meal treated with protease contained fewer antigenic proteins than the other soy-containing diets. In another study, they added alkaline protease into soybean meal and incubated for 2 h at 50˚C, pH 8.5. They found the composition of soybean meal was changed due to pretreatment, and soluble α-amino nitrogen concentrations were increased by treatment with protease. The antigenic protein concentration was reduced. Beal *et al.* (1998a, b) reported that the pre-incubation of raw soybean or soybean meal with protease significantly increased the *in vitro* nitrogen digestibility. Ghazi *et al.* (2002) pretreated soybean meal with two different proteases: one was alkaline protease (isolated from *bacillus* species) and the other one was acid protease (isolated from *Aspergillus*). Then they incorporated the soybean meal into the diets for broiler chicks. Acid protease treatment improved chick performance from 7 to 28 d of age and increased apparent ileal nitrogen digestibility and apparent nitrogen retention across the whole digestive tract. Also, enzyme pretreatment significantly reduced chick serum antisoya antibodies. They also conducted two tube-feeding experiments using pretreated soybean meal. The results showed that the acid protease treatment improved nitrogen digestibility and true metabolizable energy.

The Comparison between Optimal pH Levels of Feed-Grade Enzymes and pH Levels Found in the Crop

To date, most feed-grade enzyme products are either from fungal or from bacterial source. A pH between 4.0 and 5.0 is the optimal pH for most commercial enzymes (Beauchemin, 2003). Coughlan (1985) reported that the optimal pH for fungal cellulase is between 4.0 and 5.0. The optimal pH of fungal protease was between 3.0 and 4.7 (FCC, 1996). Two optimal pH values, 2.5 and 5.5 for microbial phytase were found (Shieh *et al.,* 1969; Simons *et al.,* 1990). Baas and Thacker (1996) measured the activities of β-glucanase at varieties pH levels using a discontinuous assay. They found that little activity was evident at pH 2.5, and the activity was only slightly increased at pH 3.5. The highest activity occurred at pH 4.5 and 5.5. Enzyme activity declined quickly when pH level was higher than 6.5. The finding of maximum β-glucanase activity with a pH between 4.5 and 5.5 in this study supports the findings of McClear and Glennie-Holmes (1985), who reported optimal activities within this range for both fungal and bacterial sources. Ademark *et al.* (2001) compared the biochemical and hydrolytic properties of four major α-galactosidase forms that were purified from the culture filtrate of *Aspergillus niger*. All enzymes had maximal activity at pH 4.5. Thacker and Baas (1996) determined pentosanase activity using ten commercial enzyme products at five pH levels. The results demonstrated that a pH below 3.5 was detrimental to enzyme activity. The enzyme activity declined rapidly when pH was more than 6.5. The maximum activity was at pH between 4.5 and 5.5. GrootWassink *et al.* (1989) also reported maximum activities were within this range for both purified and crude enzyme extracts of pentosanase.

It is generally considered that the response to exogenous carbohydrase enzymes in poultry is much better than in pigs (Chesson, 1993; Ogden, 1995; Dierick and Decuypere, 1996; Partridge, 2001). One of the major reasons for this is that poultry have a very unique organ – the crop. Instead of going directly into the acid environment of the stomach in pigs, the diet first goes into the crop in poultry where the exogenous enzymes can be active at a relatively high pH levels (Rotter *et al.*, 1989a; Bedford and Schulze, 1998; Danicke *et al.,* 1999). Herpol and Van Grembergen (1967) reported that the average pH level of the crop was 6.3. However, there was a big variation. The pH could change from minimum of 4.0 to maximum of 7.8. This is because *Lactobacilli* predominate in the crop under normal circumstances (Smith, 1965). When the feed first goes into the crop, the pH level is high. Then the pH decreases mainly due to the production

of lactic acid by the fermentation of *Lactobacilli*. Moran (1982) pointed out that the concentration of volatile fatty acids in crop content is very small in a normal situation. Jayne-Williams and Fuller (1971) reported that the lactic acid in crop digesta started increasing 3 hrs after feed consumption. Riley and Austic (1984) showed the pH of the crop content was 6.6 after feed consumption. Bolton (1965) compared the pH change of the crop content at various times after removal of the feed. The results showed that the pH of the crop content remained at 6.51 for 6 hrs after the removal of the feed.

Optimal pH levels required for feed-grade enzymes are 4.0-5.0 while the pH level found in the crop content of poultry is approximately 6.5. Thus, the high pH in the crop may be a limiting factor for maximizing the enzyme activity.

The Use of Organic Acids in Poultry Diets and Their Effects on Dietary and Digesta pH

Organic acids have been used for decades in feed preservation. Experiments with pigs have shown that several organic acids, including citric acid, fumaric acid, formic acid, and propionic acid have a positive influence on growth performance, especially for wealing pigs that often suffer from digestive disturbances resulting in diarrhea related to infections with *E. coli* (Kirchgessner and Roth, 1991; Gabert and Saucer, 1994; Partanen and Mroz, 1999). In poultry production, limited studies have been conducted to explore the effects of organic acid supplementation on the growth performance of broiler chicks. Furthermore, the results in the literature are not consistent. Vogt *et al.* (1979, 1981) reported that a positive influence on either feed conversion ratio or growth performance of broiler chicks was found for fumaric acid, propionic acid, sorbic acid and tartaric acid. Studies by Patten and Waldroup (1988) showed that addition of fumaric acid significantly increased body weight of broilers but did not influence feed utilization. Skinner *et al.* (1991) found that body weight gain, but not feed conversion of broiler chicks was improved by the supplementation of fumaric acid during a 49-day experimental period. However, Brown and Southern (1985) pointed out that addition of citric acid and ascorbic acid did not affect chick performance. Similar results were also obtained by Izat *et al.* (1990) in two experiments that studied the effects of organic acids, including formic acid, calcium formate and buffered propionic acid, in diets on the performance of broiler chicks and on microflora of the intestine and carcass. Data from Cave (1982, 1983 and 1984) indicated a depression of feed intake and growth performance by a dietary supplement of propionic acid but not lactic acid.

Dietary acidification increased gastric proteolysis and protein and amino acid digestibility in pigs (Giesting and Easter, 1985). The acidic anion has been shown to complex with Ca, P, Mg and Zn, which results in an improved digestibility of these minerals (Li *et al.,* 1998; Edwards and Baker, 1999). Shohl (1937) first reported that dietary addition of a citric acid/sodium citrate mixture to the diets that were deficient in Ca and P would prevent rickets in rats. Later, Pileggi *et al.* (1956) confirmed that citric acid had a specific effect on phytate phosphorus. Studies by Boling *et al.* (1999, 2000) showed that dietary citric acid effectively improved phytate phosphorus utilization in chicks.

A very important objective of dietary acidification is the inhibition of intestinal bacteria competing with the host for available nutrients, and a reduction of possibly toxic bacterial metabolites, *e.g.*, ammonia and amines, thus improving weight gain of the host animal (Roth and Kirchgessner, 1998; Partanen and Mroz, 1999). Furthermore, the growth inhibition of potential pathogenic bacteria and zoonotic bacteria, *e.g.*, *E. coli* and *Salmonella spp.*, in the feed and in the gastrointestinal tract are of benefit with respect to animal health. In poultry production, organic acids have been either added to drinking water to keep the watering system free from micro-organisms without causing damage to the bird or environment or used to minimize the effect of feedborne *Salmonella spp.* (Vanderwal, 1979; Broek *et al.,* 2003). Contaminated feed is one of the three major sources of *Salmonella spp.* infection in poultry. Studies showed that the problem of feedborne infection can be reduced by incorporation of formic acid into the feed (Duncan and Adams, 1972; Smyser and Snoeyenbos, 1979; Van der wal, 1979; Hinton and Linton, 1985, 1988; Humphrey and Lanning, 1988; Rouse *et al.,* 1988). In fact, organic acids exert their antimicrobial action both in the feed and in the gastrointestinal tract of the birds. Iba and Berchieri (1995) found that the acid in the diet exerted an antibacterial effect in the crop. Thompson and Hinton (1997) pointed out that the inclusion of formic acid and propionic acid in the diet of hens resulted in higher concentrations of these acids in the contents of the crop and gizzard, which were bactericidal for *Salmonella spp. in vitro*. Data from Berchieri and Barrow (1996) showed that the incorporation of a commercial formic acid preparation into poultry feed resulted in a significant reduction in incidence of experimental fowl typhoid from environmental sources. Izat *et al.* (1990) reported that dietary acidification using buffered propionic acid reduced the total number of *E. coli* in the small intestine and *Salmonella spp.* on postchill carcasses.

Organic acids are able to enter the microbial cell in the undissociated form because they are lipid soluble. Once inside the cell, the acid releases protons in the more alkaline environment, resulting in a decrease of intracellular pH (Young and Foegeding, 1993). This influences microbial metabolism inhibiting the action of important microbial enzymes and forces the bacterial cell to use energy to release protons, leading to an intracellular accumulation of acid anions. The acid anions can disrupt DNA and protein synthesis, putting the organism under stress so that it is unable to replicate or replicate rapidly (Nursey, 1997). The antibacterial activity of organic acids is related to the reduction of intracellular pH, as well as their ability to dissociate, which is determined by the pK_a value of the respective acid, and the pH of the surrounding milieu (Cherrington, 1991). The antibacterial activity increases with decreasing pH value (Russell, 1992). The lower pH conditions thus protect the animal from infection especially at young ages. Hinton *et al.* (2000) pointed out that the high amount of lactobacilli and low pH in the crop have been shown to decrease the occurrence of *Salmonella spp.* in the crop. Following dietary intake, organic acids are only recovered from foregut of the chicks (crop, gizzard and proventriculus) (Hume *et al.,* 1993). The antibacterial effect of dietary organic acids in chickens is believed to take place in the upper part of the digestive tract (crop and gizzard) (Thompson and Hinton, 1997). Its effect further down the digestive tract would diminish as concentrations decreased as a result of absorption and metabolism (Bolton and Dewar, 1964). Watkins (2004) found that the crop pH was significantly lowered by reducing the water pH. Brown and Southern (1985) reported that addition of citric acid and ascorbic acid did not affect intestinal pH of young chicks. Thompson and Hinton (1997) showed that the inclusion of formic and propionic acids to the feed of hens made no difference in the pH of the intestinal tract. In pigs, the strongest effect of organic acids with respect to digesta pH and antimicrobial activity are found in the stomach. Studies showed that the inclusion of organic acids in the diet reduced dietary and stomach pH (Kirchgessner and Roth, 1982; Falkowski and Aherne, 1984; Giesting and Easter, 1985; Radcliff *et al.* 1998).

CHAPTER 3: IN VITRO ACTIVITY EVALUATION OF FEED-GRADE ENZYME PRODUCTS AT PH LEVELS SIMULATING VARIOUS PARTS OF THE AVIAN DIGESTIVE TRACT (Study 1)

Introduction

Exogenous enzymes, such as β-glucanase [1,3-(1,3;1,4)-β-D-Glucan 3 (4)-glucanohydrolase, EC 3.2.1.6], xylanase (1,4-β-D-Xylan xylanohydrolase, EC 3.2.1.8), amylase (1,4-α-D-Glucan glucanohydrolase, EC 3.2.1.1), α-galactosidase (α-D-Galactoside galactohydrolase, EC 3.2.1.22) and protease are very commonly used feed-grade enzymes in poultry rations. Most of these enzymes are from fungal sources (Clarkson *et al.,* 2001). Studies showed that most fungal enzyme preparations have optimal activities at pH 4.0-5.0 (Ademark *et al.*, 2001; Baas and Thacker, 1996; Coughlan, 1985; Groot Wassink *et al.*, 1989; McClear and Glennie-Holmes, 1985; Thacker and Baas, 1996). The average pH levels commonly found in the poultry diet, the crop content, the gizzard and the proximal and distal small intestine are 6.0, 6.5, 3.0, 7.0 and 7.5 respectively (Bolton, 1965; Herpol and Van Grembergen 1967; Riley and Austic 1984; William and Richard, 1984). Studies by Baas and Thacker (1996) showed that very little activity was evident for both β-glucanase and xylanase when the pH level was below 3.5 and the enzyme activity decreased dramatically when the pH level was higher than 6.5. Based on these results, the pH levels in chicken gastrointestinal tract may be either too low (in the gizzard) or too high (in the crop and small intestine) for maximizing the activities of the feed-grade enzymes.

Objective

This *in vitro* study was conducted to measure the activities of feed-grade enzyme products at pH levels simulating various parts of the avian digestive tract.

Materials and Methods

Materials

Five commercial enzyme preparations including β-glucanase, xylanase, amylase, α-galactosidase and protease, all from fungal sources, were used. The enzymes were supplied by

Alltech Inc., Nicholasville, KY, USA. All other chemicals were of analytical grade and purchased from Sigma Chemical Co., St. Louis, MO, USA, or Fisher Scientific Inc., Pittsburgh, PA, USA.

pH and Buffers

Six pH levels were used in this study. One pH level was the optimum pH level for each enzyme referred from publications or from FCC (1996). The other five pH levels, including 3.0, 6.0, 6.5, 7.0 and 7.5, were used to simulate pH levels found in the gizzard, the diet, the crop, and the proximal and distal parts of small intestine, respectively. The pH gradient was obtained by dissolving the enzyme and substrate in different buffers. The buffers used were as follows: pH 3.0, 5.3 and 6.0 (0.05M) - Na-citrate buffer; pH 4.8 and 5.0 (0.1 M) – Na-acetate buffer; pH 6.5, 7.0 and 7.5 (0.1 M) – Phosphate buffer or N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer (only for amylase).

Measurement of Enzyme Activity

A 1.0 ± 0.0001g sample of each enzyme product was weighted in triplicate and extracted in 100 ml of the appropriate buffer, followed by stirring for 15 min using an electromagnetic mixer and stir bar. The samples stood for 15 min after stirring. A 0.5~1.0 ml of the supernatant was taken and diluted to the required concentration using the same buffer.

The activity of β-glucanase was determined according to the method of Miller (1959) as modified by Bathgate (1979). Beta-glucan from barley (Sigma G-6513, St. Louis, MO) was used as a substrate. Reducing sugar (glucose) was determined after 10 min incubation at 30°C. One β-glucanase activity unit (BGU) was defined as that quantity of enzyme that liberates 1 μmol of reducing sugars (expressed as glucose) in 1 min under the assay conditions (Appendix 1).

The activity of xylanase was measured according to the method of Bailey and Poutanen (1989). This assay was based on measurement of reducing sugar (xylose) following a 5 min hydrolysis of xylan substrate (Sigma X-0502, St. Louis, MO) at 50°C. One xylanase activity unit (XU) was defined as the amount of enzyme that liberates 1 μmol of xylose in 1 min under the conditions of the assay (Appendix 2).

A method from FCC (1996) was used to assay α-amylase. Potato soluble starch (Fisher S-516-100, Fair Lawn, NJ) was used as a substrate. An iodine solution was added to the buffered starch solution to produce blue color. Alpha-amylases break down the α-1-4 glucosidic linkages of the starch to yield maltose and smaller dextrins. As starch is broken down, the color changes

from blue to red-brown. The color produced was compared to a standard color solution. The enzyme activity was expressed as fungal amylase unit (FAU), with 1 unit equal to the amount of enzyme that dextrinized soluble starch at the rate of 1 mg per min at 30°C (Appendix 3).

Alpha-galactosidase was assayed according to the procedure described by Ratto and Poutanen (1988). The assay was based on a 5 min hydrolysis of ρ-nitrophenyl-α-D-galactoside (PNPG, Sigma N-0877, St. Louis, MO) at 40°C followed by spectrophotometric measurement of the liberated ρ-nitrophenol at 405 nm. One galactosidase activity unit (GalU) was defined as the quantity of the enzyme that liberates ρ-nitrophenol at the rate of 1 μmol/min under the conditions of the assay (Appendix 5).

The method from FCC (1996) was used to determine the activity of protease. The test was based on a 30-min enzymatic hydrolysis of a Hammarsten-grade casein (United States Biochemical Corp, Cat #12840, Cleveland, OH) substrate at 37°C. Unhydrolyzed substrate was precipitated with trichloroacetic acid (TCA) and removed by filtration. The quantity of released tyrosine in the filtrate was determined through measuring the absorbance spectrophotometrically at 275 nm. The activity of the enzyme was expressed as spectrophotometric acid protease unit (SAP) with 1 unit equal to the amount of enzyme that liberates 1 μmol of tyrosine per min from casein under the conditions specified (Appendix 4).

Results

The activity of β-glucanase, xylanase, amylase, α-galactosidase and protease at various pH levels are presented in Figures 3.1, 3.2, 3.3, 3.4 and 3.5, respectively. Beta-glucanase (Figure 3.1) had high activity from pH 3.0 to 6.5. The enzyme activity decreased when the pH level was higher than 6.5.

The highest activity of xylanase occurred from pH 5.3 to 6.5 (Figure 3.2). The enzyme activity was only 7% of the activity at optimum pH (5.3) when the pH was lowered to 3.0. The enzyme activity declined dramatically when the pH was higher than 6.5.

The maximum activity of fungal α-amylase was observed at pH 4.8 (Figure 3.3). No activity was observed at pH 3.0 for this enzyme and the activity decreased dramatically when the pH was above 6.5.

Alpha-galactosidase had highest activity at pH 5.0 (Figure 3.4). Little activity was observed when the pH was lowered to 3.0. A quick reduction of activity was found when the pH was higher than 6.0.

The highest activity of protease was observed at pH 3.0 (Figure 3.5). The enzyme activity decreased very quickly when the pH was higher than 5.5.

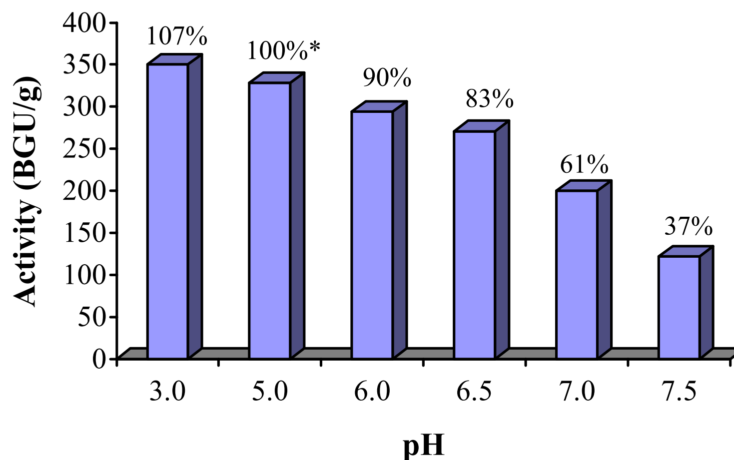

Figure 3.1. Activity of β-glucanase at different pH levels expressed as β-glucanase units (BGU) per g enzyme product. Values on top of bars represent activity as a percentage of activity obtained at optimum pH (5.0)

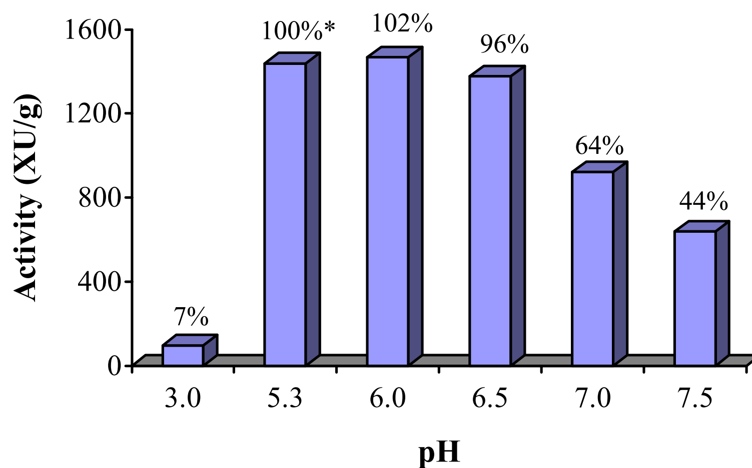

Figure 3.2. Activity of xylanase at different pH levels expressed as xylanase units (XU) per g enzyme product. Values on top of bars represent activity as a percentage of activity obtained at optimum pH (5.3)

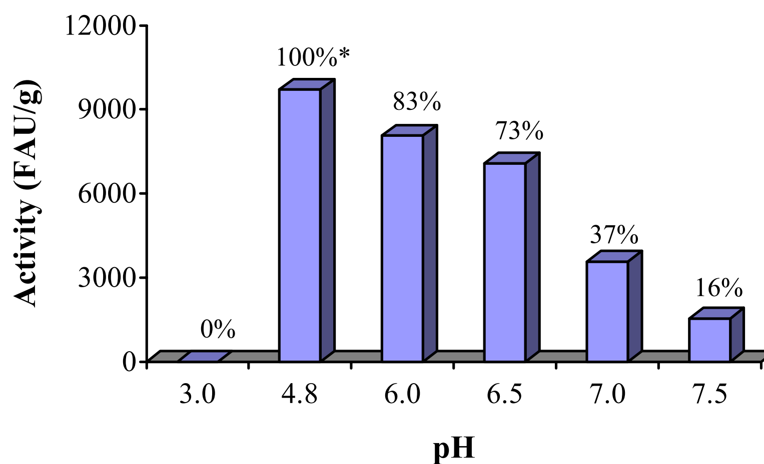

Figure 3.3. Activity of fungal amylase at different pH levels expressed as fungal amylase units (FAU) per g enzyme product. Values on top of bars represent activity as a percentage of activity obtained at optimum pH (4.8)

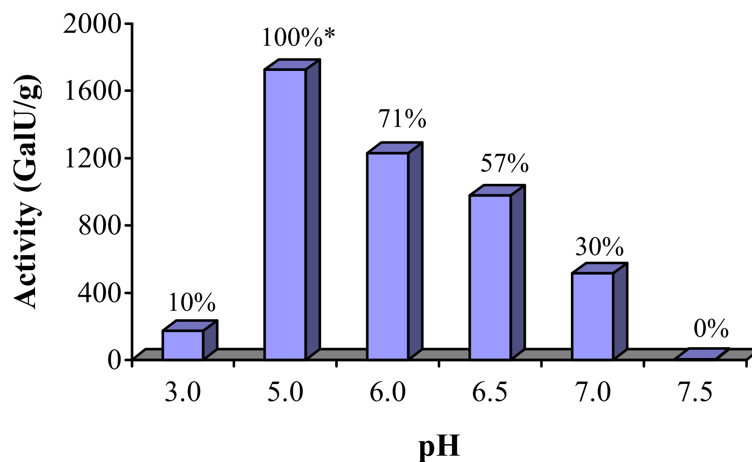

Figure 3.4. Activity of α-galactosidase at different pH levels expressed as galactosidase units (GalU) per g enzyme product. Values on top of bars represent activity as a percentage of activity obtained at optimum pH (5.0)

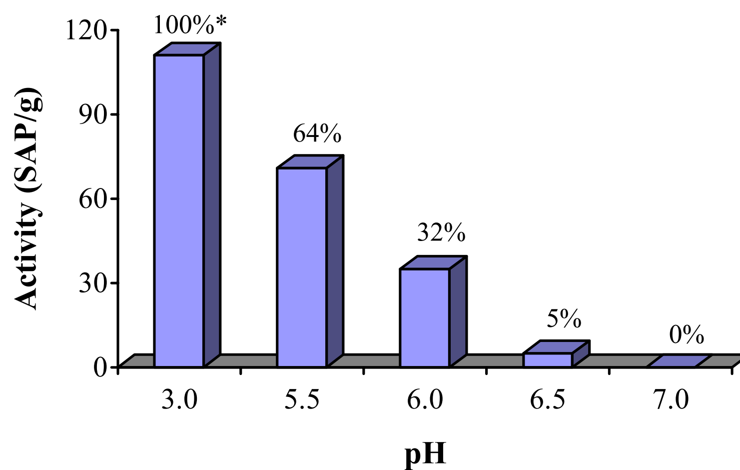

Figure 3.5. Activity of fungal protease at different pH levels expressed as spectrophotometric acid protease units (SAP) per g enzyme product. Values on top of bars represent activity as a percentage of activity obtained at optimum pH (3.0)

Discussion

Miller (1959) and Bathgate (1979) reported that the optimal pH level of β-glucanase was 5.0. Similar results were also observed by McClear and Glennie-Holmes (1985). Baas and Thacker (1996) pointed out that the highest activity of β-glucanase occurred at pH 4.5 and 5.5 and enzyme activity declined dramatically at pH 6.5 or above. These results are consistent with the results found in this study. However, this study did not show any reduction of activity at pH 3.0 compared with that at pH 5.0, which is opposite to the results reported by Baas and Thacker (1996) who showed little activity at pH 3.0 for several commercial fungal β-glucanase products.

The data found in this study about the activity of xylanase at different pH levels supports the results obtained by Thacker and Baas (1996), Bailey and Poutanen (1989) and GrootWassink *et al.* (1989). Based on their data, Thacker and Baas (1996) reported that little activity of feed-grade pentosanase was evident when pH was lower than 3.5, while the enzyme activity was significantly lower at pH higher than 6.5. Bailey and Poutanen (1989) found the optimum pH of xylanase was 5.3. GrootWassink *et al.* (1989) showed that the maximum activity was at pH 5.0 for both purified and crude enzyme extracts of pentosanase.

The results obtained from this study showed that the optimal pH of amylase was around 5.0. This is similar to the findings reported by Sandsted *et al*. (1939) and Farrand (1964) and the optimal pH level of 4.8 for the activity assay of fungal α-amylase suggested by FCC (1996).

Ratto and Poutanen (1988) found the optimal pH of α-galactosidase was 5.3. The pH level for the activity measurement of α-galactosidase suggested by FCC (1996) is 5.5. These data are consistent with the finding obtained from this study. However, Ademark *et al.* (2001) compared the biochemical and hydrolytic properties of four major fungal α-galactosidase forms that were purified from the culture filtrate of *Aspergillus niger*. They found that all enzymes had maximal catalytic activity at pH 4.5.

The optimal pH level of protease found in this study is 3.0. This is the same as that proposed in FCC (1996).

The data from this study suggested that the optimal pH level for four of the five enzymes assayed (β-glucanase, xylanase, amylase and α-galactosidase, but not protease) was around 5.0 and all the enzymes had reduced activity at pH 6.5 or above. These results are in agreement with other published data and the data found in FCC (1996). The pH levels commonly found in

different segments of the avian digestive tract may be a limiting factor for maximum activity of the exogenous enzymes such as amylase, α-galactosidase and protease.

CHAPTER 4: EFFECTS OF SUPPLEMENTING ORGANIC ACIDS IN THE DIET ON THE DIETARY AND DIGESTA PH AND GROWTH PERFORMANCE OF BROILER CHICKS (Study 2)

Introduction

Studies with pigs have indicated a positive response in growth performance to dietary additions of various organic acids (Falkowski and Aherne, 1984; Geisting and Easter, 1985; Patten and Waldroup, 1988; Skinner *et al.,* 1991). However, the results of studies examining growth performance of chickens following the dietary supplementation of organic acids are not as convincing as those from pigs (Langhout, 2000). Some studies showed a positive influence on either weight gain or feed conversion ratio, or both, following dietary inclusion of organic acids such as fumaric acid or propionic acid (Vogt *et al.,* 1979, 1981; Patten and Waldroup 1988; Skinner *et al.* 1991). But other studies showed either no effect (Brown and Lee Southern, 1985; Izat *et al.,* 1990) or showed a negative effect on growth performance by supplementing the same kind of organic acids (Cave, 1982, 1983 and 1984).

There is ample evidence that organic acids have antimicrobial action (Cherrington *et al.,* 1991; Russell, 1992; Nursey, 1997). In poultry production, organic acids are mainly used to sanitize feed and water to inhibit pathogens, such as *Salmonella spp.* (Van der wal, 1979; Broek *et al.,* 2003). In recent years, there has been an increase in the use of acidifiers as substitutes of growth promoters due to the concern about the consequences of feeding antibiotics to livestock on both human and animal health (Martin and Williams, 2002). Studies have suggested that the addition of organic acids influences concentration of bacteria in the ceca and small intestine (Vogt *et al.,* 1981); that it is bactericidal for *Salmonella spp.* in the diet and crop (Duncan and Adams, 1972; Smyser and Snoeyenbos, 1979; Van der wal, 1980; Hinton and Linton, 1988; Humphrey and Lanning, 1988; Rouse *et al.,* 1988; Iba and Berchieri, 1995; Thompson and Hinton, 1997) and that it reduces the prevalence of *Salmonella spp.* on the carcass (Van der wal, 1980; Hinton *et al.,* 1985; Rouse *et al.,* 1988; Izat *et al.,* 1990).

Barley and wheat are generally considered to contain high levels of soluble non-starch polysaccharides (NSPs) (White *et al.*, 1981; Choct and Annison, 1990). Soluble NSPs possess anti-nutritive activity in poultry, which is partially related to the gut microflora (Annison and

Choct, 1991). Increasing the amount of soluble NSPs can increase the microbial load in the small intestine (Choct *et al.*, 1996). This negative effect of NSPs was corrected by using antibiotics (Moran *et al.*, 1969; MacAuliffe and McGinnis, 1971). Dietary inclusion of organic acids in barley or wheat containing diets may diminish the negative effects of NSPs through its antimicrobial action.

A study on the metabolism of dietary propionic acid revealed that the majority (75%) of propionic acid was used as an energy source (Hume *et al.*, 1993). Bolton and Dewar (1964) also pointed out that organic acids served as substrates in the intermediary metabolism. This suggests a relationship between energy levels of the diet and the effect of the organic acids.

It is well documented that the dietary inclusion of organic acids lowers the dietary and stomach pH in the pigs (Kirchgessner and Roth, 1982; Falkowski and Aherne, 1984; Giesting and Easter, 1985; Radcliff *et al.*, 1998). However, there is limited information on the influence of dietary additions of organic acids on the pH of the diet and the digesta in poultry.

Objectives

The general objectives of this study were 1) to evaluate the effects of dietary supplementation of organic acids on the pH of the diet (Experiment 2.1); 2) to investigate the effects of dietary addition of citric acid on the pH of the diet and the digesta and on the growth performance of broiler chicks fed different grain sources (Experiment 2.2); and 3) to compare the effects of citric acid and fumaric acid on the growth performance of broiler chicks fed corn-soybean meal-based diet with different energy levels (Experiment 2.3).

Materials and Methods

Organic Acids

Food grade anhydrous citric acid was obtained from Roche Vitamins Inc., Parsippany, NJ. Fumaric acid was purchased from Kic Chemicals, Inc., Armonk, NY. Propionic anhydride (97%), acetic acid (99.7%) and formic acid (95~97%) were from Aldrich Chemical Company, Inc., Milwaukee, WI.

Animal Care

The animal care and use protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

Experiment 2.1

Experimental Design and Dietary Treatments. This experiment was designed to evaluate the effects of adding graded levels of organic acids to the diet on the pH of the diet. Five different organic acids, including formic acid, acetic acid, propionic acid, fumaric acid and citric acid, at six different levels (0–5%) were mixed with a corn-soybean meal-based diet in triplicate batches using 5 x 6 factorial arrangement. The composition of the basal diet is listed in Table 4.1. The diet was mixed using a M20MK-1 Mixer (Univex Corporation, Salem, NH). Organic acids replaced the corn in the diet. The dietary pH was determined immediately after mixing according to the method described by Radcliffe *et al.* (1998). Five grams of diet was added to 50 ml deionized water and stirred for 1 min using an electromagnetic mixer and stir bar. The Accumet®BASIC AB15 pH meter with combination electrode (Fisher Scientific Inc., Pittsburgh, PA) was used to directly measure the pH of the solution while stirring.

Experiment 2.2

Experimental Design and Animals. This experiment was conducted to study the effects of dietary supplementation of citric acid on the pH of the diet and the digesta and on the growth performance of broiler chicks fed different grain sources. Three hundred sixty 1-d-old male broiler chicks were obtained from Avian Division, Cobb-Vantress, Monticello, KY. The chicks were housed in mesh wire-floored standard pullet starter cages (61 cm x 51 cm x 36 cm) in an environmentally controlled room. Continuous light was provided for 22 h/d. Each cage had one feeder that was removable for weighing and two adjustable nipple drinkers. Feed and water were supplied on an *ad libitium* basis. The temperature in the room was 31°C for the first week and then lowered to 27°C for the remainder of the study. The experiment used a randomized complete block design. Blocks were based on the cage locations within a room. Each experimental unit consisted of one cage of six birds. Six replicate cages were assigned to each of ten dietary treatments. Dietary treatments were randomly distributed to cages within each of six blocks.

Dietary treatments consisted of feeding 1) a corn basal diet (CB); 2) CB + 0.5% citric acid; 3) CB + 1% citric acid; 4) CB + 1.5% citric acid; 5) CB + 2% citric acid; 6) CB + 2.5% citric acid; 7) a barley basal diet (BB); 8) BB + 1.5% citric acid; 9) a wheat basal diet (WB) or 10) WB + 1.5% citric acid. Three basal diets (CB, BB and WB) contained an energy level that was similar to that recommended for commercial production (Classic and HI-Y Broiler Management

Guide, HUBBARD farms, 2004). Other nutrients were formulated based on the requirements of broilers as established by National Research Council (NRC) (1994). The ingredient composition and the calculated nutrient analysis of the basal diets are given in Table 4.1 and Table 4.2, respectively. The experiment lasted 3 wk. Bird mortality was monitored daily. If a bird died within the first 4 days, it was replaced by a bird of similar weight from an extra group of chicks receiving the same diet. Birds and feed were weighed initially and then weekly. After each week, average body weight gain, average daily feed intake and gain to feed ratio were calculated. On Day 16, four birds from each treatment were killed by asphyxiation with argon gas followed by cervical dislocation. The digesta from the crop, gizzard and small intestine were collected separately. The pH were immediately determined after collection. On Day 23, a total of 60 birds (10 birds for each treatment) from Treatment 1, 4, 7, 8, 9 and 10 were not given feed for 12 h. Then, the birds were fed for 20 min before the feeders were removed. Digesta from the crop, gizzard and small intestine were collected separately at 0, 0.5, 1.0, 1.5, and 2.0 h after the removal of the diet. The pH was immediately determined after collection. The method used in measuring diet pH was also used for the determination of digesta pH.

Experiment 2.3

Experimental Design and Animals. This experiment was designed to compare the effects of citric acid and fumaric acid on the growth performance of broiler chicks fed corn-soybean meal-based diets with different energy levels. Two hundred sixteen 1-d-old male broiler chicks were used in this experiment. The chick supplier and the housing conditions were exactly the same as in the previous experiment. The experiment used a randomized complete block design with three blocks based on the cage locations within a room. A 2 x 3 factorial arrangement of treatments was used. The factors consisted of two energy levels and three acid sources. Dietary treatments were randomly assigned to cages within each block.

Table 4.1. Ingredient composition of basal diets (Experiment 2.1 and 2.2)

Ingredients	Corn basal	Barley basal	Wheat basal
	(%)		
Corn	58.00	–	–
Barley	–	59.69	–
Wheat	–	–	61.00
Soybean meal (48%CP)	35.90	30.80	31.00
Corn oil	2.10	5.70	3.98
Salt	0.45	0.45	0.45
Limestone	1.32	1.35	1.37
Dicalcium phosphate	1.76	1.53	1.70
DL-Methionine	0.22	0.23	0.25
Vitamin-mineral mix[1]	0.25	0.25	0.25

[1]Supplied per kg diet: 11,025 I.U. vitamin A, 3,528 I.U. vitamin D_3, 33 I.U. vitamin E, 0.91 mg vitamin K, 2 mg thiamin, 8 mg riboflavin, 55 mg niacin, 18 mg Ca pantothenate, 5 mg vitamin B-6, 0.221 mg biotin, 1 mg folic acid, 478 mg choline, 28 µg vitamin B-12, 75 mg zinc, 40 mg iron, 64 mg manganese, 10 mg copper, 2 mg iodine and 0.3 mg selenium.

Table 4.2. Nutrient composition of basal diets (Experiment 2.1 and 2.2)[1]

Nutrient	Corn basal diet	Barley basal diet	Wheat basal diet
AME_n, Mcal/kg	3.01	3.02	3.03
CP, %	22.00	22.08	22.04
Fat, %	4.44	7.14	5.82
Fiber, %	2.62	2.69	3.04
Ca, %	1.00	0.98	1.00
Available P, %	0.45	0.45	0.46
Methionine, %	0.54	0.55	0.56
Methionine + cystine, %	0.92	0.90	0.91
Lysine, %	1.24	1.16	1.13
Sodium, %	0.20	0.20	0.22

[1]Values reported are calculated and "as-is" basis.

The experimental diets used were 1) a low energy basal diet (2740 Kcal/kg) (LB); 2) LB + 2% citric acid; 3) LB + 2% fumaric acid; 4) a normal energy basal diet (3010 Kcal/kg) (NB); 5) NB + 2% citric acid; and 6) NB + 2% fumaric acid. The normal energy corn-soy basal diet was the same as the diet used in the previous studies (Tables 4.1 and 4.2). The LB was formulated to contain 9% less energy level than NB. The composition of the ingredient and the calculated nutrient analysis of the low energy basal diet is given in Table 4.3. The experiment lasted two weeks. Bird mortality was monitored daily. If a bird died within the first 4 days, it was replaced by a bird of similar weight from an extra group of chicks receiving the same diet. Birds and feed were weighed initially and then weekly. After each week, average body weight gain, average daily feed intake and gain to feed ratio were calculated.

Table 4.3. Ingredient and nutrient composition of low energy basal diet (Experiment 2.3)

Ingredients	Diet, %	Nutrients	Calculated analysis
Corn	52.00	AME_n, Mcal/kg	2.74
Soybean meal (48%CP)	36.00	CP, %	22.97
Alfalfa (17%CP)	8.40	Fat, %	2.34
Salt	0.44	Fiber, %	4.52
Limestone	1.06	Ca, %	1.00
Dicalcium phosphate	1.68	Available P, %	0.45
DL-Methionine	0.17	Methionine, %	0.53
Vitamin-mineral mix[1]	0.25	Methionine + cystine, %	0.89
		Lysine, %	1.28
		Sodium, %	0.20

[1]Supplied per kg diet: 11,025 I.U. vitamin A, 3,528 I.U. vitamin D_3, 33 I.U. vitamin E, 0.91 mg vitamin K, 2 mg thiamin, 8 mg riboflavin, 55 mg niacin, 18 mg Ca pantothenate, 5 mg vitamin B-6, 0.221 mg biotin, 1 mg folic acid, 478 mg choline, 28 µg vitamin B-12, 75 mg zinc, 40 mg iron, 64 mg manganese, 10 mg copper, 2 mg iodine and 0.3 mg selenium.

Statistical Analysis

Data were subjected to ANOVA for a 5 x 6 factorial design in Experiment 2.1 or a randomized complete block design in Experiment 2.2 and Experiment 2.3 using the linear model of Statistix V.8. (2003) (Analytical Software, Tallahassee, FL). Mean differences were determined using Fisher's least significant difference test. Significance was declared when probability was less than 5%. Linear and quadratic effects were tested using polynomial contrasts. Orthogonal contrasts (Snedector and Cochran, 1989) were used in Experiment 2.2 to identify differences between grain sources and the addition of citric acid. The coefficients for the contrasts are outlined in Table 4.4.

Table 4.4. Coefficients for treatment contrasts (Experiment 2.2)

Contrast description	Dietary treatment coefficient					
	1	2	3	4	5	6
	Corn basal (CB)	CB +1.5% citrate	Barley basal (BB)	BB +1.5% citrate	Wheat basal (WB)	WB +1.5% citrate
Grain source (1 & 2 vs. 3 & 4)	1	1	-1	-1	0	0
Grain source (1 & 2 vs. 5 & 6)	1	1	0	0	-1	-1
Grain source (3 & 4 vs. 5 & 6)	0	0	1	1	-1	-1
Citric acid supplementation (1, 3 & 5 vs. 2, 4 & 6)	1	-1	1	-1	1	-1

Results

Experiment 2.1

The effects of different sources and levels of organic acids on dietary pH are presented in Table 4.5. Dietary pH was cubically reduced with increasing levels of organic acids in the diet (Figure 4.1) (P<0.001). A significant interactive effect was found between acid levels and acid sources (P<0.001). At the same acid level, different acids have different effects on the dietary pH. The ability of organic acids in lowering the pH of the diet was as follows: formic acid > citric acid > fumaric acid > acetic acid > propionic acid.

Experiment 2.2

Dietary supplementation of citric acid linearly reduced the pH of the diet and the crop content (P<0.001), but not the pH of the gizzard digesta and small intestine digesta (Table 4.6). After removal of the diet, the pH of the crop digesta remained at high levels (around 6.0-6.5) up to 2 h for the birds fed CB, BB or WB control diet (Table 4.7). The crop content sampled from the birds fed diets containing citric acid had lower pH values than that sampled from the birds fed basal diets. Weight gain, feed intake and weight to gain ratio of broiler chicks during three week experimental period were not affected by including graded levels of citric acid in the corn-

soybean meal-based diet (Table 4.8). Supplementing citric acid in the basal diets containing different grain sources had no significant effect on growth performance of broiler chicks (Table 4.9).

Experiment 2.3

The effects of citric acid and fumaric acid on the growth performance of broiler chicks fed a corn-soybean meal-based diet with different energy levels are presented in Table 4.10. The chicks fed normal energy level diets had significantly ($P<0.01$) higher weight gain and gain to feed ratio than those fed low energy diets. Weight gain during Day 1-7 was depressed by fumaric acid. Negative effects on weight gain and gain to feed ratio during Day 1-14 were produced by both acids. Feed intake was unaffected by adding acid to the diet. There was no significant difference between citric acid and fumaric acid regarding the growth performance depression. No interactive effects between energy levels and acid sources were observed.

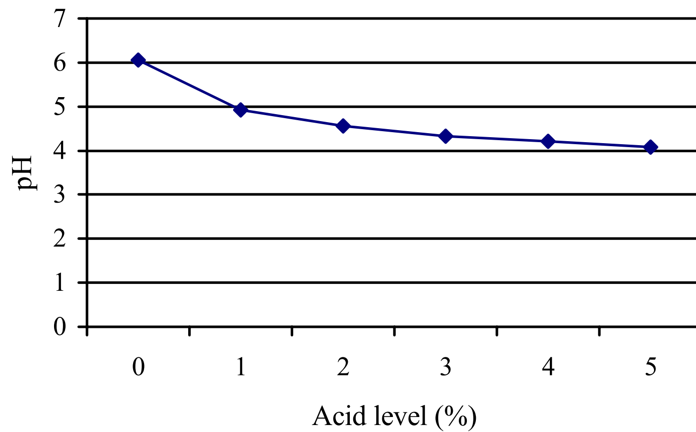

Figure 4.1. Effects of different levels of organic acid on dietary pH

Table 4.5. Effects of different levels and sources of organic acids on dietary pH (Experiment 2.1)

	Acid source				
Acid level, %	Formic acid	Citric acid	Fumaric acid	Acetic acid	Propionic acid
	pH[1]				
0	6.09	6.04	6.04	6.01	6.07
1	4.45	5.08	5.00	4.96	5.17
2	4.00	4.67	4.61	4.63	4.90
3	3.79	4.30	4.43	4.53	4.63
4	3.65	4.09	4.35	4.42	4.53
5	3.50	4.03	4.13	4.32	4.42

Main effect means

Acid level, %	Diet pH	Acid source	Diet pH
0	6.05	Propionic acid	4.96[a]
1	4.93	Acetic acid	4.81[b]
2	4.56	Fumaric acid	4.76[c]
3	4.33	Citric acid	4.70[d]
4	4.21	Formic acid	4.25[e]
5	4.08		

Source of variation[2]	(P)	Linear	Quadratic	Cubic
Level	0.001	0.001	0.001	0.001
Source	0.001			
Level x source	0.001			

[1]Values are means of two measurements from triplicate samples.
[2]Error mean square = 0.002.
[a, b, c, d, e] Within a column, means without a common superscript letter differ, $P<0.01$.

Table 4.6. Effects of supplementation of citric acid on the pH of the diet and digesta (Experiment 2.2)[1]

Treatment	Diet	Crop	Gizzard	Small Intestine
	pH			
Corn-soy basal (CB)	6.29	6.19	3.03	7.38
CB + 0.5% citric acid	5.55	5.87	3.03	7.34
CB + 1.0% citric acid	5.30	5.62	3.34	7.39
CB + 1.5% citric acid	5.04	5.33	3.13	7.11
CB + 2.0% citric acid	4.57	5.13	3.23	7.29
CB + 2.5% citric acid	4.48	4.96	3.10	7.22
SEM[2]	0.01	0.12	0.15	0.19
Sources of variation	(P)			
Linear	0.001	0.001	1.00	0.98
Quadratic	0.001	0.98	0.94	1.00

[1]Data presented are means of four birds.

[2]Standard error of the mean.

Table 4.7. Crop digesta pH at different time intervals after removal of the diet (Experiment 2.2)[1]

Diet	Sample time after removal of diet (h)				
	0	0.5	1.0	1.5	2.0
	pH				
Corn basal (CB)	6.25	6.38	6.26	6.22	NA
CB + 1.5% citrate	5.30	5.45	5.57	NA	4.51
Barley basal (BB)	6.08	6.16	6.11	6.30	6.04
BB + 1.5% citrate	4.73	5.09	5.33	5.34	5.34
Wheat basal (WB)	6.32	6.32	6.47	6.32	6.18
WB + 1.5% citrate	4.8	5.22	5.34	5.36	5.50

[1]Data presented are means of two birds for each of six replicates.

Discussion

Dietary addition of organic acids significantly (P<0.01) lowered the pH of the diets and the crop content but did not change the pH of the gizzard and the small intestine. Giesting and Easter (1985) showed a significant reduction in dietary pH by inclusion of citric acid, fumaric acid and propionic acid. Both citric acid and fumaric acid caused a greater reduction in dietary pH than did propionic acid. Thompson and Hinton (1997) reported that the inclusion of the organic acids in the diet of the hen caused the accumulation of the acids in the crop, but did not change the small intestine pH. Brown and Southern (1985) pointed out that citric acid addition did not affect intestinal pH of young chicks.

Either no effects (Table 4.8) or negative effects (Table 4.10) on growth performance of broiler chicks by supplementing citric acid and fumaric acid were observed in this study. These data support the results obtained by Izat *et al.* (1990) and Brown and Southern (1985) who reported that citric acid addition did not affect chick growth performance. The depression of growth performance by supplementing propionic acid was noticed by Cave (1984). However,

studies by Vogt *et al.* (1979, 1981) showed that the addition of fumaric acid in the diet up to 2% significantly increased weight gain and gain to feed ratio.

This study did not show significant interactions between grain sources and organic acid supplementation. It is probably because the barley and wheat used in this study did not contain high amounts of NSPs. The content of NSPs in barley and wheat can vary among varieties (Aastrup and Much, 1985; Annison and Choct, 1993; Bamfort 1982). Other factors, *e.g.*, growing conditions, can also affect NSPs concentrations. In the present study, the birds fed barley or wheat basal diet had the same or even better growth performance than those fed corn basal diet (Table 4.9). Therefore, it is not surprising that adding citric acid to the barley or wheat basal diet did not result in improved performance.

Table 4.8. Effects of dietary supplementation of graded levels of citric acid on growth performance of broiler chicks fed corn-soy basal diet (Experiment 2.2)[1]

Diet	Weight gain (g/bird)			Feed intake (g/bird)			Gain to feed ratio (g/g)		
	1 – 7 d	1 – 14 d	1 – 21 d	1 – 7 d	1 – 14 d	1 – 21 d	1 – 7 d	1 – 14 d	1 – 21 d
Corn basal (CB)	106	360	759	135	483	1025	0.78	0.75	0.74
CB + 0.5% citrate	114	374	775	144	491	1032	0.79	0.76	0.75
CB + 1.0% citrate	110	369	771	138	482	1026	0.80	0.77	0.75
CB + 1.5% citrate	105	348	718	129	466	987	0.82	0.75	0.73
CB + 2.0% citrate	106	353	751	133	468	998	0.80	0.75	0.75
CB + 2.5% citrate	115	376	783	139	490	1047	0.82	0.77	0.75
SEM[2]	2.45	6.68	12.76	2.99	8.22	13.73	0.13	0.01	0.01
Source of variation	(P)								
Linear	0.98	1.00	1.00	0.97	0.99	1.00	0.36	0.98	1.00
Quadratic	0.96	0.85	0.61	0.94	0.82	0.43	1.00	1.00	1.00

[1]Data presented are means of six groups of six birds.

[2]Standard error of the mean.

Table 4.9. Effects of different grain sources and citric acid supplementation on growth performance of broiler chicks (Experiment 2.2)[1]

Diet		Weight gain (g/bird)			Feed intake (g/bird)			Gain to feed ratio (g/g)		
		1 – 7 d	1 – 14 d	1 – 21 d	1 – 7 d	1 – 14 d	1 – 21 d	1 – 7 d	1 – 14 d	1 – 21 d
1	Corn basal (CB)	106	360	759	135	483	1025	0.78	0.75	0.74
2	CB + 1.5% citrate	105	348	718	129	466	987	0.82	0.75	0.73
3	Barley basal (BB)	107	365	779	130	479	1063	0.83	0.76	0.73
4	BB + 1.5% citrate	107	371	788	132	477	1059	0.82	0.78	0.74
5	Wheat basal (WB)	117	382	804	133	477	1044	0.87	0.80	0.77
6	WB + 1.5% citrate	117	372	795	140	481	1041	0.83	0.77	0.76
	SEM[2]	2.49	8.49	14.18	3.31	9.04	15.13	0.02	0.01	0.01
Orthogonal contrasts[3]										
Grain source (1 & 2 vs. 3 & 4)		ns	ns	ns	ns	ns	*	ns	ns	ns
Grain source (1 & 2 vs. 5 & 6)		**	ns	**	ns	ns	ns	ns	*	**
Grain source (3 & 4 vs. 5 & 6)		*	ns	ns	ns	ns	ns	ns	ns	*
Citric acid supplementation (1, 3 & 5 vs. 2, 4 & 6)		ns	ns	ns	ns	ns	ns	ns	ns	ns

[1]Data presented are means of six replicate groups of six birds.
[2]Standard error of the mean.
[3]Significance of contrast: ns = not significant, * = P < 0.05, ** = P < 0.01.

Table 4.10. The effects of citric acid and fumaric acid on growth performance of broilers fed diets with different energy levels (Experiment 2.3)[1]

Treatment		Weight gain (g/bird)		Feed intake (g/bird)		Gain to feed ratio (g/g)	
		1 – 7d	1 – 14d	1 – 7d	1 – 14d	1 – 7d	1 – 14d
Energy	Acid						
Low		81	294	159	528	0.51	0.56
Normal		97	346	162	515	0.60	0.68
	No acid	94[a]	340[a]	166	510	0.57	0.67[a]
	+ citrate	88[ab]	306[b]	164	533	0.55	0.58[b]
	+ Fumarate	83[b]	313[b]	152	521	0.55	0.60[b]
Low	No acid	83	309	162	516	0.51	0.60
Low	+ citrate	81	283	162	528	0.51	0.54
Low	+ Fumarate	77	289	152	540	0.51	0.54
Normal	No acid	105	372	168	504	0.63	0.74
Normal	+ citrate	95	329	165	538	0.59	0.62
Normal	+ Fumarate	90	337	153	503	0.59	0.67
EMS[2]		86	458	246	1327	0.006	0.004
Sources of variation		Significance of treatment effect[3]					
Energy		**	**	ns	ns	**	**
Acid		*	**	ns	ns	ns	**
Energy x acid		ns	ns	ns	ns	ns	ns

[1]Six replicate groups of six birds were assigned to each of the six treatments.
[2]Error mean square.
[3]Significance of treatment effect: ns = not significant; * = P<0.05; ** = P<0.01.
[a, b] Means within a column that lack a common superscript letter differ.

CHAPTER 5: IN VITRO EVALUATION OF SIMULTANEOUS SUPPLEMENTATION OF CITRIC ACID AND EXOGENOUS ENZYMES ON NUTRIENT RELEASE FROM SOYBEAN MEAL AND TRYPSIN INHIBITOR CONTENT IN RAW WHOLE SOYBEAN (Study 3)

Introduction

The majority of protein in animal feeds is supplied by vegetable proteins, with the soybean meal (SBM) being the main provider. However, the availability of nutrients in SBM is often limited by the presence of anti-nutritional factors (ANFs) (Classen *et al*., 1993). The major ANFs in SBM include trypsin inhibitors, lectins and galacto-oligosaccharides (GOS) (verbascose, stachyose and raffinose) (Huisman and Jansman, 1991). Soybean meal contains roughly 6% GOS which cannot be digested by monogastric animals, such as poultry and pigs, because of the absence of endogenous enzymes with α-(1,6)-galactosidase activity (Gitzelmann and Auricchio, 1965). Undigested GOS can negatively influence growth rates and protein digestion of young chicks and pigs (Veldman *et al.,* 1993; Gdala *et al.,* 1997) and cause flatulence (Rachis *et al.,* 1970). Exogenous α-galactosidase can be used to alleviate the anti-nutritional effects of GOS in soybean meal (Marsman *et al.,* 1997; Graham *et al.,* 2002). Alpha-galactosidase can hydrolyze GOS into sucrose and galactose, which can be further digested and utilized by animals. Heat treatment has proved most effective at reducing levels of trypsin inhibitors and lectin in soybean meal (Lalles, 1993). However, commercial heat processing is carefully controlled. Underheating can result in inadequate inactivation of ANFs while overheating may reduce availability of nutrients (especially lysine) through the occurrence of Maillard reactions (Huisman and Tolman, 1992). Therefore, treatment of SBM with exogenous protease to inactivate proteinaceous anti-nutritional factors is a potential method for improving the nutritional value of SBM without the undesirable effects of heat treatment (Classen *et al*., 1993). In addition, the application of protease in animal diets can increase the digestion of the large storage protein molecules in soybean meal that cannot be efficiently digested and utilized by young animals due to the underdeveloped digestive system (Sheppy, 2001).

The optimum pH level of fungal α-galactosidase is *ca*. 4.5 (Ademark *et al.,* 2001) while that of protease is around 3.0 (FCC, 1996). A typical corn-soybean meal-based diet for broiler

chicks has a pH of approximately 6.0. The addition of organic acids, *e.g*., citric acid, is known to lower dietary pH (Giesting and Easter, 1985).

Objective

The objective of this experiment was to test the hypothesis that acidification of diet (soybean meal or raw whole soybean) containing exogenous enzymes will increase the enzyme activity through optimizing the pH of the digesta.

Materials and methods

Materials

Commercial preparations of α-galactosidase and fungal protease (Alltech Inc., Nicholasville, KY) were used in this experiment. Pepsin (P7012) and pancreatin (P 3292) were purchased from Sigma Chemical Co., St Louis, MO. All other chemicals used were of analytical grade. Soybean meal containing 48% crude protein and raw whole soybean were obtained from commercial suppliers. Food grade anhydrous citric acid was purchased from Roche Vitamins Inc., Parsippany, NJ.

Measurement of Enzyme Activity

Activity of α-galactosidase was determined based on the method described by Ratto and Poutanen (1988). One galactosidase unit (GalU) was defined as the quantity of the enzyme that liberates ρ-nitrophenol at the rate of 1 µmol/min under the conditions of the assay. The activity of α-galactosidase used in this study was 1724 GalU/g. Activity of protease was assayed according to the method from FCC (1996). One spectrophotometric acid protease unit (SAP) is the amount of enzyme that liberates 1 µmol of tyrosine per min from casein under the conditions specified. The activity of protease measured was 111 SAP/g.

Experimental Treatments

Substrates, experimental factors, *in vitro* measurement, phase of digestion and statistical design performed in Experiments 3.1-3.6 are presented in Table 5.1. Substrates included SBM and raw whole soybean (RWSB). The RWSB was defatted using petroleum ether before use in the *in vitro* digestion process. To do so, the soybean was ground in a coffee grinder to a fine texture and put in a filter bag. The bag was sealed and soaked into petroleum ether. The ether

was changed every three or four hours until it was clear. Then, the excess ether was removed and the bags were spread out in the hood allowing ether to evaporate.

In Vitro Digestion Procedure and pH Levels in Simulating Digestive Tract

A modified *in vitro* digestion procedure described by Tervila-Wilo *et al.* (1996) and Zyla *et al.* (1999) was used in this study and is presented in Figure 5.1 and Figure 5.2. In these procedures, the pH level of the crop digesta was modified to the dietary pH level, which was based on the previous study results (Experiment 2.2) and simulated the pH of the crop content at the initial residence of the diet. The pH levels used for simulating other parts of the digestive tract were the same as those used by Tervila-Wilo *et al.* (1996). Reducing sugars, α-amino nitrogen and trypsin inhibitor content were determined after incubation.

In Vitro Digestion and Measurement of Reducing Sugars and α-Amino Nitrogen

Triplicate samples of substrates were ground through a 1 mm screen and then weighed 2.5 ± 0.0001g into 50 ml centrifuge tubes. The samples were hydrated with 6 ml distilled water and 1 ml of 1% (w/v) enzyme solution. The contents of each tube were vortexed, and then the tubes were sealed with parafilm and incubated in a water bath at 40°C for 30 min. The first step simulated the digestion in the crop. If only the crop phase digestion was considered, 5 ml ice-cold distilled water was added to the tube to stop the hydrolysis. Otherwise, 1.5-2.0 ml of 1 M HCl solution (based on pH = 3.0) and 7500 units (0.5 ml) of pepsin solution were added to each tube, vortexed and sealed with parafilm and reincubated for 45 min at the same temperature. This step simulated digestion in proventriculus and gizzard of chickens. If only crop through gizzard digestion was considered, 3 ml ice-cold distilled water was added to the tube to stop the reaction. Otherwise, 3 ml $NaHCO_3$ containing 4.63 mg pancreatin was added drop wise with constant stirring to each tube. The tubes were vortexed, sealed with parafilm and further incubated for another 60 min. This step simulated digestion in small intestine. Once the digestion process was finished, the tubes were immediately put in an ice-cold water bath for 15 min to stop the further hydrolysis. Then, the tubes were centrifuged at 16,240 x g for 20 min. The supernatants (0.5 ml) were withdrawn and treated with 0.3 N barium hydroxide and 0.3 N zinc sulfate solutions to precipitate protein as described by Sonnenwirth and Jarett (1980). The deproteinization was completed through centrifuging at 7676 x g for 10 min at 20°C. Deproteinized samples were used either to measure reducing sugars using method by Miller *et al.* (1959) (Appendix 7) or to

Table 5.1. Experimental factors, *in vitro* analysis, phase of digestion and statistical design for Experiment 3.1-3.6

Experiment	Substrate	Experimental factor	*In vitro* analysis	Phase of digestion	Statistical analysis
3.1.	SBM	Citric acid (0, 2%) α-Galactosidase (0, 5172, 6896, 8620, 10344, 12068, 13792 GalU/kg)	Reducing sugar	Whole[1]	2 x 7 factorial ANOVA
3.2.	SBM	Citric acid (0, 2%) α-Galactosidase (0, 1724, 3448, 5172, 6896, 8620 GalU/kg)	Reducing sugar	Crop	2 x 6 factorial ANOVA
3.3.	SBM	Citric acid (0, 2%) Protease (0, 222, 444, 666, 888 SAP/kg)	α-Amino nitrogen	Crop	2 x 5 factorial ANOVA
3.4.	SBM	Citric acid (0, 2%) Protease (0, 222, 333, 444, 555 SAP/kg)	α-Amino nitrogen	Whole	2 x 5 factorial ANOVA
3.5.	RWSB	Citric acid (0, 2%) Protease (0, 222, 333, 444, 555 SAP/kg)	α-Amino nitrogen	Gizzard[2]	2 x 5 factorial ANOVA
3.6.	RWSB	Citric acid (0, 2%) Protease (0, 222, 333, 444 SAP/kg)	Trypsin inhibitor	Gizzard	2 x 4 factorial ANOVA

[1]From crop through small intestine.
[2]From crop through gizzard.

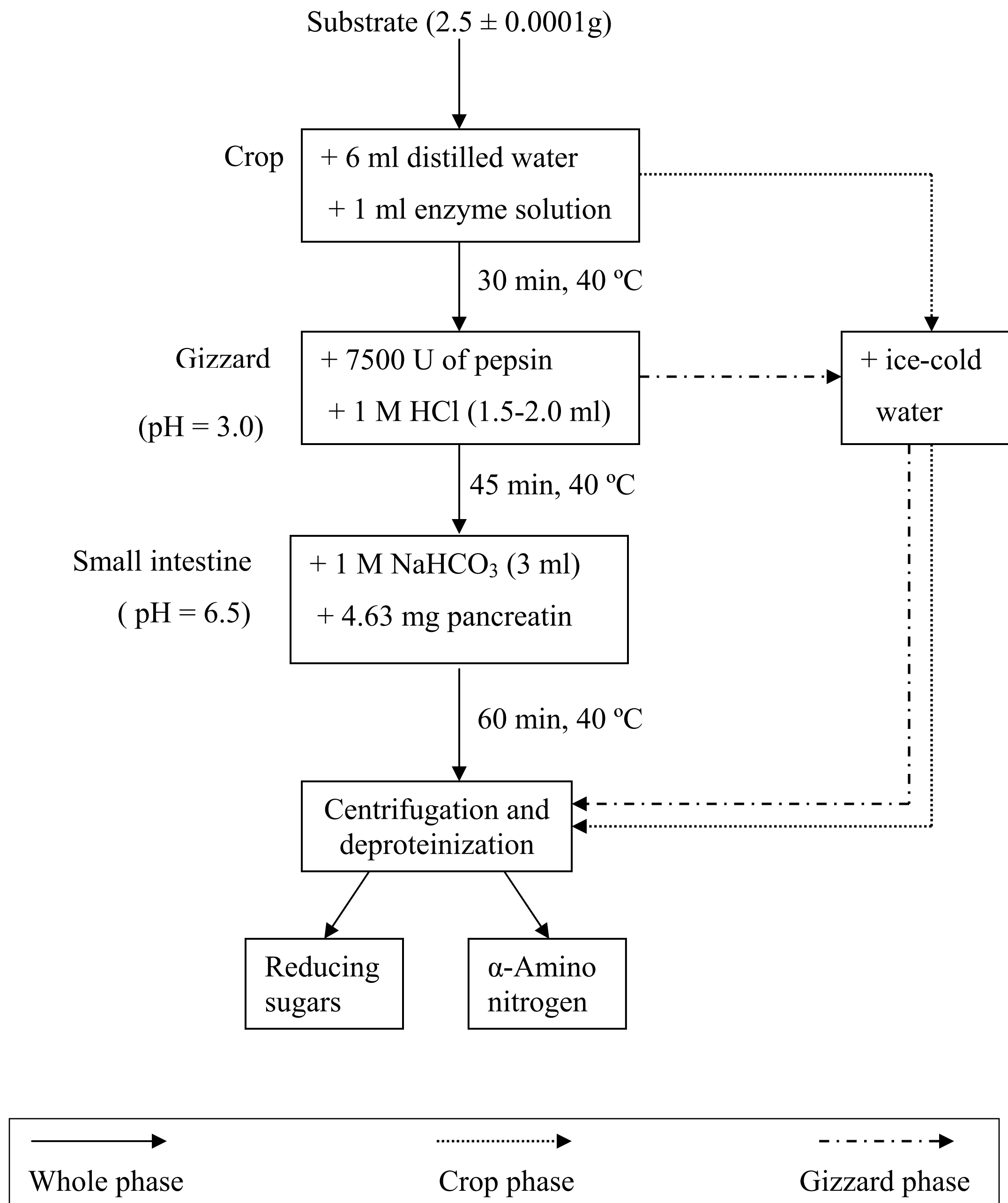

Figure 5.1. Flow chart of *in vitro* digestion procedure designed for the determination of reducing sugars and α-amino nitrogen (modified from Zyla *et al.,* 1999 and Tervila-Wilo *et al.,* 1996)

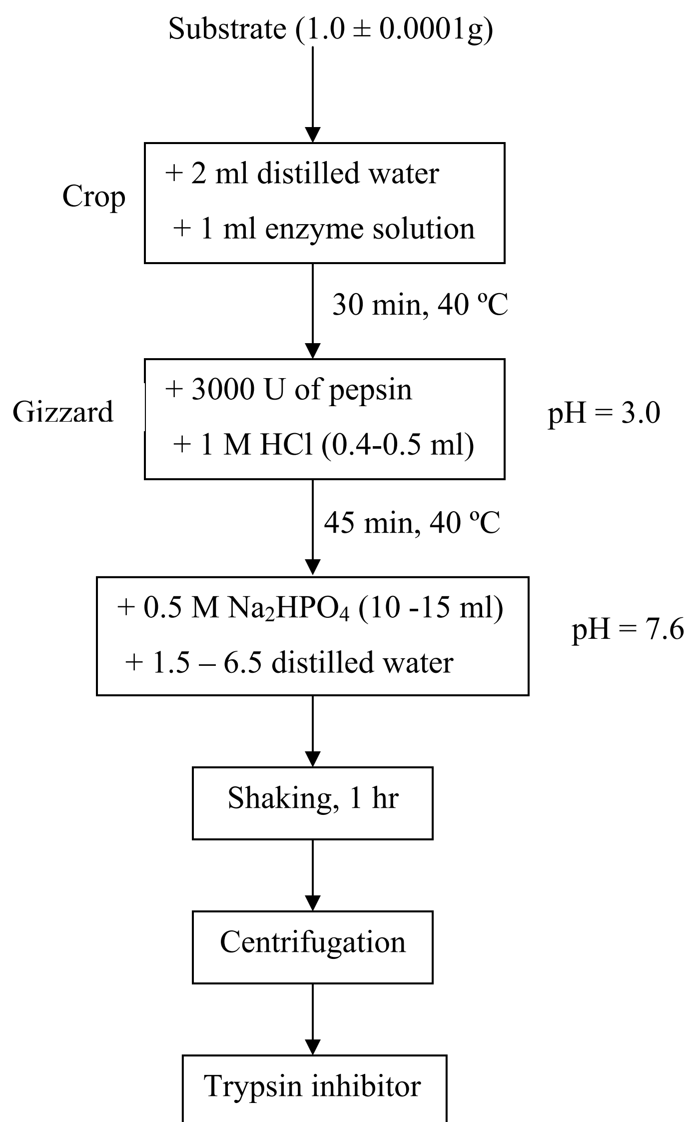

Figure 5.2. Flow chart of *in vitro* digestion procedure designed for the determination of trypsin inhibitor content (modified from Zyla *et al.,* 1999 and Tervila-Wilo *et al.,* 1996)

measure α-amino nitrogen with a method by Moore and Stein (1954) (Appendix 6).

In Vitro Digestion and Measurement of Trypsin Inhibitor Content

Triplicate samples of defatted RWSB were ground through a 1 mm screen and then weighed 1.0 ± 0.0001g into 50 ml centrifuge tubes. The samples were hydrated with 2 ml distilled water and 1 ml of 1% (w/v) enzyme solution. The content of each tube was vortexed, and then the tubes were sealed with parafilm and incubated in a water bath at 40°C for 30 min. The first step simulated the digestion in the crop. Then, 0.4–0.5 ml of 1.0 M HCl solution (based on pH = 3.0) and 3000 units (0.5 ml) of pepsin solution were added to each tube, vortexed and sealed with parafilm and reincubated for 45 min at the same temperature. This step simulated the digestion in the proventriculus and the gizzard of chickens. Once the digestion process was finished, the tubes were immediately put in an ice-cold water bath for 15 min to stop the further hydrolysis. Then, 10–15 ml Na_2HPO_4 (0.5 M) was added to each tube to adjust the pH of the solution to 7.6. Distilled water (1.5-6.5 ml) was also added to each tube to adjust the total volume of the solution to 20.5 ml. The tubes were vortexed, sealed with parafilm and continuously shaken in a mechanical shaker for 1 hr. Then, the tubes were centrifuged at 16,240 x g for 20 min. The supernatants were withdrawn and used to analyze the trypsin inhibitor content according to a procedure proposed by Kunitz (1947), as modified by Kakade *et al.* (1969) (Appendix 8). One trypsin unit (TU) is arbitrarily defined as an increase of 0.01 absorbance units at 280 nm in 20 min per 10 ml of the reaction mixture under the assay conditions. Trypsin inhibitor activity is defined as the number of trypsin units inhibited (TUI).

Statistical Analysis

Data were collected from nine replicates and analyzed by the general linear model procedure (GLM) of Statistix V.8 (2003) (Analytical Software, Tallahassee, FL). Results were subjected to analysis of variance following the model:

$$X_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ij}$$

where μ is the overall mean, α_i denotes the first-factor effect, β_j denotes the second-factor effect, $(\alpha\beta)_{ij}$ represents the interaction between factors and e_{ij} is the error contribution. Mean differences were determined using Fisher's least significant difference test. Statistical significance was accepted at $P<0.05$. Polynomial contrasts were used to determine linear and quadratic effects of enzymes.

Results

The effects of α-galactosidase and citric acid on reducing sugar release from SBM in the whole phase digestion (Experiment 3.1) are presented in Table 5.2. The addition of α-galactosidase significantly ($P<0.001$) increased the amount of reducing sugars. The inclusion of citric acid further increased ($P<0.001$) the enzyme activity. A significant interaction ($P<0.001$) between α-galactosidase and citric acid was found. The release of reducing sugars increased linearly ($P<0.001$) as more enzyme was included in the incubation mixture no matter the citric acid was included or not included (Figure 5.3). However, the two lines have different slopes. At same enzyme level, more reducing sugars were released for the treatments with citric acid than those without citric acid.

The effects of α-galactosidase and citric acid on reducing sugar release from SBM in the crop phase digestion (Experiment 3.2) are presented in Table 5.3 and Figure 5.4. Similar results to those observed in the whole phase digestion were found in the crop phase.

The effects of protease and citric acid supplementation on α-amino nitrogen release from SBM and RWSB during simulated digestion through the crop, whole and gizzard phases are listed in Tables 5.4, 5.5 and 5.6 respectively. In all three phases, the addition of protease to the substrates significantly increased ($P<0.001$) the α-amino nitrogen release. The linear effect of protease supplementation on α-amino nitrogen release from SBM in the whole digestion phase and from RWSB in the gizzard digestion phase are shown in Figure 5.5 and Figure 5.6, respectively. Addition of citric acid significantly increased ($P<0.05$) the release of α-amino nitrogen in both the whole and gizzard phases. A significant interaction between protease and citric acid was observed in the crop phase (Table 5.4). Enzyme activation was noticed in crop phase when citric acid was included, but not excluded in SBM (Figure 5.7). Neither protease nor citric acid had effect on the trypsin inhibitor content in RWSB (Table 5.7).

Table 5.2. Effects of α-galactosidase and citric acid on release of reducing sugars from soybean meal in the whole phase (Experiment 3.1)[1]

Enzyme (GalU/kg)	No citric acid	+ Citric acid
	Reducing sugars (g/kg)	
0	7.8	7.6
5172	20.7	27.0
6896	24.8	31.8
8620	29.7	38.8
10344	34.6	44.9
12068	36.0	49.3
13792	39.4	61.1

Main effect means

Enzyme (GalU/kg)	Reducing sugars (g/kg)	Citric acid, %	Reducing sugars (g/kg)
0	7.7	0	27.6
5172	23.8	2	37.2
6896	28.3		
8620	34.2		
10344	39.7		
12068	42.6		
13792	50.2		

Source of variation[2]	(P)	Linear
Enzyme	0.001	0.001
Citric acid	0.001	
Enzyme x citric acid	0.001	

[1]Values are means of three samples, analyzed in duplicate.
[2]Error mean square = 8.50.

Table 5.3. Effects of α-galactosidase and citric acid on release of reducing sugars from soybean meal in the crop phase (Experiment 3.2)[1]

Enzyme (GalU/kg)	No citric acid	+ Citric acid
	Reducing sugars (g/kg)	
0	0.0	0.0
1724	6.3	7.0
3448	9.4	13.4
5172	13.9	17.0
6896	17.1	23.1
8620	19.1	26.9

Main effect means

Enzyme (GalU/kg)	Reducing sugars (g/kg)	Citric acid, %	Reducing sugars (g/kg)
0	0.0	0	10.9
1724	6.6	2	14.6
3448	11.4		
5172	15.4		
6896	20.1		
8620	23.0		

Source of variation[2]	(P)	Linear
Enzyme	0.001	0.001
Citric acid	0.001	
Enzyme x citric acid	0.001	

[1]Values are means of three samples, analyzed in duplicate.
[2]Error mean square = 1.27.

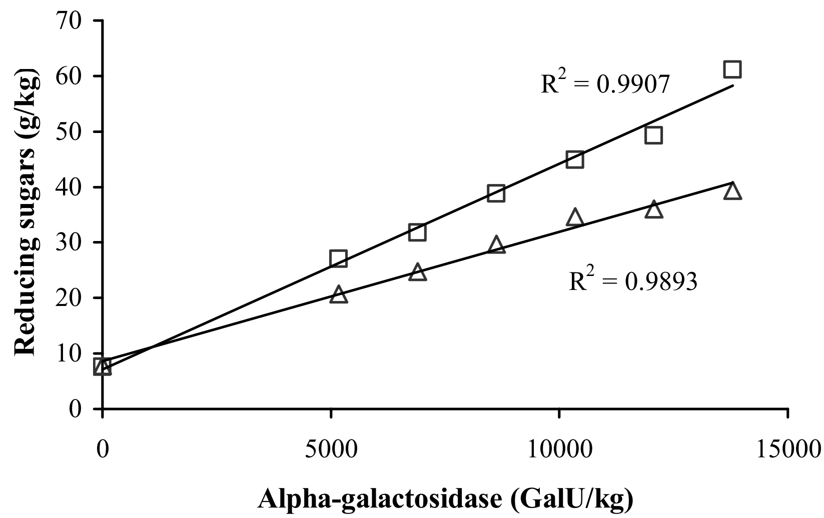

Figure 5.3. The release of reducing sugars from SBM by dietary α-galactosidase in the whole phase. □ = + citric acid; Δ = no citric acid. Two linear lines have been fitted to the data to demonstrate the difference in slope between the lines and hence the dietary α-galactosidase by citric acid interaction (Experiment 3.1).

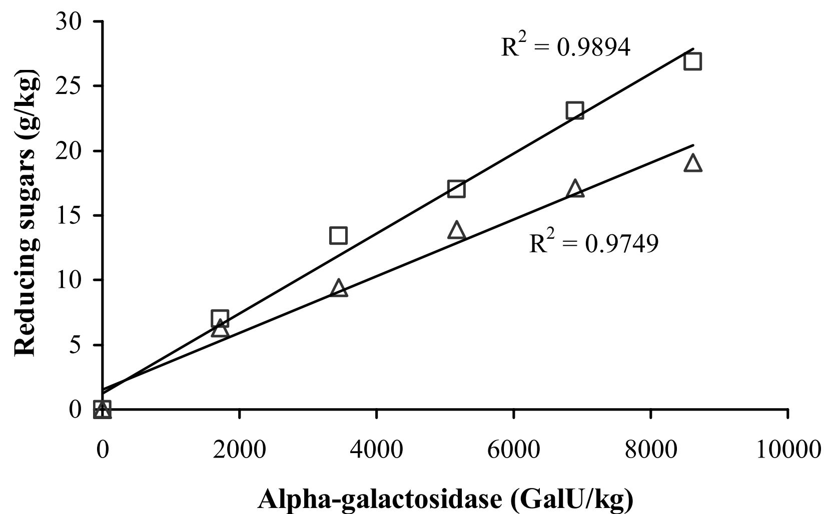

Figure 5.4. The release of reducing sugars from SBM by dietary α-galactosidase in the crop phase. □ = + citric acid; Δ = no citric acid. Two linear lines have been fitted to the data to demonstrate the difference in slope between the lines and hence the dietary α-galactosidase by citric acid interaction (Experiment 3.2).

Table 5.4. Effects of protease and citric acid on release of α-amino nitrogen from soybean meal in the crop phase (Experiment 3.3)[1]

Protease (SAP/kg)	No citric acid	+ Citric acid
	α-Amino nitrogen (mg/kg)	
0	218	381
222	247	407
333	222	379
444	275	405
555	227	510

Main effect means

Protease (SAP/kg)	α-Amino nitrogen (mg/kg)	Citric acid, %	α-Amino nitrogen (mg/kg)
0	300	0	238
222	327	2	416
333	300		
444	340		
555	368		

Source of variation[2]	(P)	Linear
Enzyme	0.001	0.001
Citric acid	0.001	
Enzyme x citric acid	0.001	

[1]Values are means of three samples, analyzed in duplicate.
[2]Error mean square = 0.0004.

Table 5.5. Effects of protease and citric acid on release of α-amino nitrogen from soybean meal in the whole phase (Experiment 3.4)[1]

Protease (SAP/kg)	No citric acid	+ Citric acid
	α-Amino nitrogen (g/kg)	
0	1.36	1.46
222	1.48	1.51
444	1.44	1.59
666	1.54	1.57
888	1.57	1.62

Main effect means

Protease (SAP/kg)	α-Amino nitrogen (g/kg)	Citric acid, %	α-Amino nitrogen (g/kg)
0	1.41	0	1.48
222	1.50	2	1.55
444	1.52		
666	1.56		
888	1.60		

Source of variation[2]	(P)	Linear
Enzyme	0.047	0.001
Citric acid	0.020	
Enzyme x citric acid	0.755	

[1]Values are means of three samples, analyzed in duplicate.
[2]Error mean square = 0.008.

Table 5.6. Effects of protease and citric acid on release of α-amino nitrogen from raw whole soybean in the gizzard phase (Experiment 3.5.)[1]

Protease (SAP/kg)	No citric acid	+ Citric acid
	α-Amino nitrogen (g/kg)	
0	1.36	1.62
222	1.37	1.82
333	1.40	1.86
444	1.51	1.85
555	1.56	1.92

Main effect means

Protease (SAP/kg)	α-Amino nitrogen (g/kg)	Citric acid, %	α-Amino nitrogen (g/kg)
0	1.49	0	1.44
222	1.59	2	1.81
333	1.63		
444	1.68		
555	1.74		

Source of variation[2]	(P)	Linear
Protease	0.001	0.001
Citric acid	0.001	
Protease x citric acid	0.191	

[1]Values are means of three samples, analyzed in duplicate.
[2]Error mean square = 0.007.

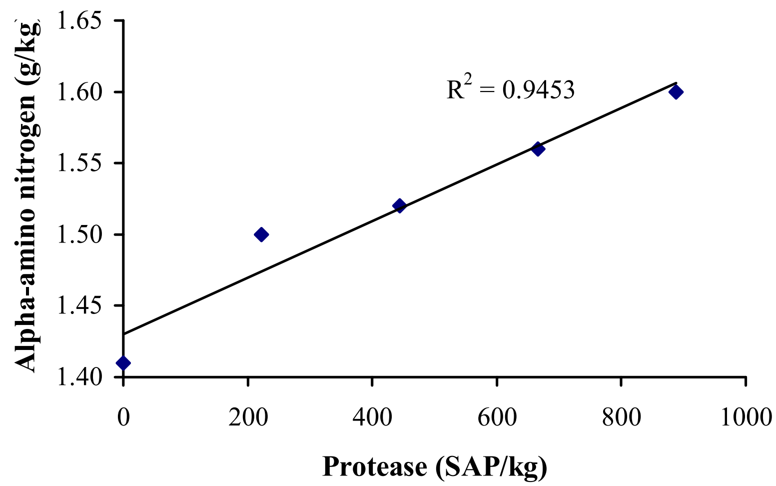

Figure 5.5. Linear effect of protease supplementation on α-amino nitrogen release from soybean meal in the whole phase

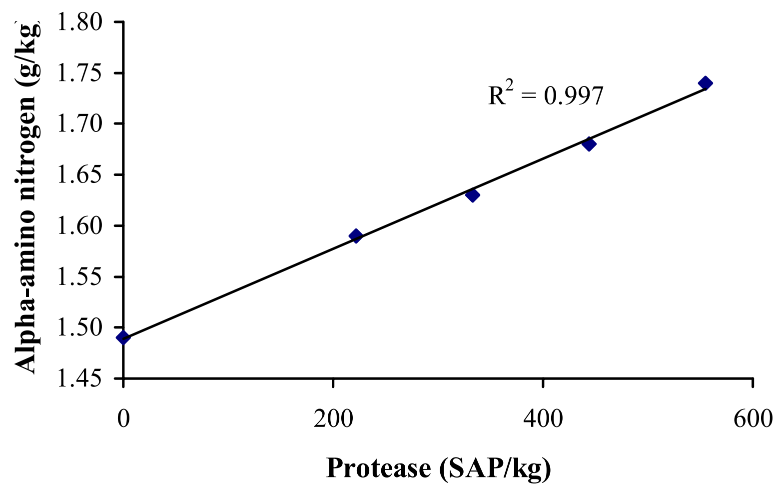

Figure 5.6. Linear effect of protease supplementation on α-amino nitrogen release from raw whole soybean in the gizzard phase

Table 5.7. Effects of protease and citric acid on trypsin inhibitor content in raw whole soybean after *in vitro* digestion (Experiment 3.6)[1]

Protease (SAP/kg)	No citric acid	+ Citric acid
	Trypsin inhibitor content (TUI/mg)	
0	19.7	20.4
222	20.5	19.7
333	20.6	18.2
444	21.1	21.1

Main effect means

Protease (SAP/kg)	Trypsin inhibitor content (TUI/mg)	Citric acid, %	Trypsin inhibitor content (TUI/mg)
0	20.0	0	20.5
222	20.1	2	19.9
333	19.4		
444	21.1		

Source of variation[2]	(P)
Protease	0.291
Citric acid	0.252
Protease x citric acid	0.276

[1]Values are means of three samples, analyzed in duplicate.
[2]Error mean square = 1.86.

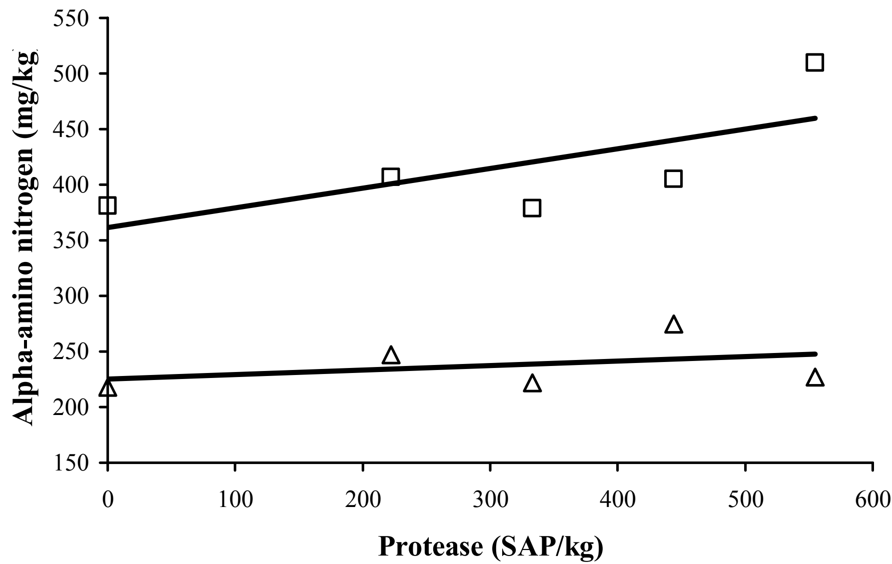

Figure 5.7. The release of α-amino nitrogen from SBM by dietary protease in the crop phase. □ = + citric acid; Δ = no citric acid. Two linear lines have been fitted to the data to demonstrate the difference in slope between the lines and hence the dietary protease by citric acid interaction (Experiment 3.3).

Discussion

Soybean meal contains approximately 6% galacto-oligosaccharides that can be hydrolyzed into galactose (reducing sugar) and fructose by α-galactosidase (Gitzelman and Auricchio, 1965; Trugo *et al.,* 1995). Graham et al (2002) reported that the α-galactosidase treatment of SBM hydrolyzed 69% of raffinose and 54% of stachyose. These results support the findings obtained in this study. In Experiment 3.1 and 3.2, the reducing sugar release from SBM linearly increased in both crop and whole digestion phases by including graded levels of α-galactosidase.

The significant improvement in sugar release due to citric acid indicates that the pH level in the crop phase is a limiting factor for maximizing the activity of α-galactosidase. The pH levels for the SBM with and without citric acid addition were 5.0 and 6.5, respectively. This result is backed by previously presented data (Study 1) that showed that α-galactosidase had only about 50% activity at pH 6.5 compared with its activity at pH 5.0. These results are also consistent with those reported by Ratto and Poutanen (1988) and Ademark *et al.* (2001).

At the same enzyme level, more reducing sugars were released in the whole phase than in the crop phase (Tables 5.2 and 5.3). This means that the low pH (3.0) level found in gizzard and host proteolytic enzymes (pepsin and pancreatin) are not detrimental to this enzyme, even though very low (10% of maximum) enzyme activity was evident at pH 3.0 as demonstrated by the previous study (Study 1). Chesson (1993) pointed out that fungal polysaccharidases are not attacked *in vitro* by the major porcine digestive proteases used singly or in combination. Baas and Thacker (1996) studied the capability of different β-glucanase products to recover activity after pre-incubation for 15, 30, 60 and 120 min at low pH levels. They found that pre-incubation at pH 3.5 caused loss of activity, with greater loss at pH 2.5. However, some enzyme activity was recovered upon return to pH 5.5. In another very similar study, they found that pentosanases treated at pH 3.5 only partially recovered activity when returned to pH 5.5 and all enzymes exhibited a serious loss of activity when incubated at pH 2.5 and then returned to pH 5.5.

Data from Experiments 3.3, 3.4 and 3.5 showed that the α-amino nitrogen concentration was linearly increased by including graded levels of protease in the substrates. These results are in agreement with the data reported by Rook *et al.* (1998) who found that the pretreatment of SBM with proteases changed the composition of the SBM, and soluble α-amino nitrogen concentrations were increased. Beal *et al.* (1998a) also found approximately 5-12% increases of *in vitro* nitrogen digestibility of raw soybean and processed soybean meal by pre-incubation with the proteases. In another study, Beal *et al.* (1998b) observed a significant reduction in the number and density of protein bands of SBM pretreated with protease, indicating the hydrolysis of storage proteins.

An interaction between protease and citric acid in the crop phase means that the pH level in the crop is a limiting factor for the activity of this enzyme at this site. Actually, protease had no enzyme activity at all when the SBM without inclusion of citric acid was incubated in the crop phase (Table 5.4). In this situation, the pH of the crop content was approximately 6.5. The previous study (Study 2) showed that the activity of this protease at pH 6.5 was only 5% of the activity at optimal pH level (3.0).

In Experiments 3.4 and 3.5, α-amino nitrogen release in the whole and gizzard phases was increased by adding citric acid in the substrate (Tables 5.5 and 5.6). This is probably due to the enhancement of the activities of pepsin or pancreatic proteases.

Although increasing levels of protease resulted in a linear increase in the release of α-amino nitrogen from RWSB (Figure 5.6), it did not reduce the concentration of trypsin inhibitor content (Table 5.7.). Huo *et al.* (1993) reported that the bacterial proteases appeared to be more effective at breaking down trypsin inhibitors than the fungal proteases.

CHAPTER 6: EFFECT OF DIETARY ENZYMES AND ACIDIFICATION OF DIET AND WATER ON NUTRIENT DIGESTIBILITY AND GROWTH PERFORMANCE OF BROILER CHICKS (Study 4)

Introduction

Corn and soybean meal are the most important energy and protein sources in poultry diets. Although the gross energy of soybean meal exceeds the gross energy of corn, its metabolizable energy is less than that of corn. This is partially due to the content of α-galactosides (raffinose and stachyose) that cannot be digested in the small intestine of monogastric animals due to the absence of endogenous α-galactosidase (Gitzelmann and Auricchio, 1965). The accumulation of these oligosaccharides in the alimentary tract may result in fluid retention and an increased flow-rate of digesta that could adversely affect the digestion and absorption of nutrients (Wiggins, 1984). Supplementation of poultry diets with exogenous α-galactosidase is one efficient approach to help animals hydrolyze these oligosaccharides (Kidd *et al.,* 2001; Graham *et al.,* 2002). Corn is generally considered to be highly digestible, especially with respect to carbohydrates. However, there is evidence to suggest that the supplementation of corn-based diets with enzymes, such as amylase, has beneficial effects on animal performance (Gracia *et al.,* 2003).

Studies with chicks and pigs have indicated a positive response of growth performance to dietary addition of various organic acids (Vogt *et al.,* 1981; Falkowski and Aherne, 1984; Geisting and Easter, 1985; Patten and Waldroup, 1988; Skinner *et al.,* 1991). The mechanism by which organic acids improve performance in broiler chicks has not been clearly determined. However, several studies have suggested that the addition of organic acids influences the concentration of bacteria in the ceca and small intestine (Vogt *et al.,* 1981); that it is bactericidal for salmonellae in the crop (Thompson and Hinton, 1997) and that it reduces the incidence of salmonellae on the carcass (van der Wal, 1980; Hinton *et al.*, 1985; Rouse *et al.,* 1988; Izat *et al.,* 1990).

In recent years, there has been an increase in the use of acidifiers as substitutes of antibiotic growth promoters due to the concern of the development of microbial resistance (Radcliffe,

2000; Martin and Williams, 2002). Acidification of water has been considered to be one of the most effective methods to sanitize drinking water (Broek *et al.,* 2003).

The optimal pH level of many commercial fungal α-galactosidases and amylases is approximately 5.0 (Farrand, 1964; Ademark *et al.,* 2001) while a typical corn-soybean meal-based diet for broiler chicks has a pH of approximately 6.0. The previous studies showed that dietary inclusion of organic acid could reduce the pH of the diet and crop digesta (Experiment 2.2) and enhance the activity of α-galactosidase *in vitro* (Experiments 3.1 and 3.2).

Proteolytic hydrolysis of endogenous protease and pH levels are two major factors that influence exogenous enzyme activity in the GI tract (Chesson, 1993). Birds have a very unique organ – the crop. Two major functions of the crop are storing and moistening the food. It is also an ideal place for an exogenous enzyme to hydrolyze a substrate that cannot be digested by the bird itself. This is because the crop has no endogenous enzyme secretion and the crop pH can be adjusted with dietary changes (Experiment 2.2).

Increasing the amount of feed intake will decrease the rate of passage of feed from the crop (Heuser, 1945), *i.e.*, feed will reside in the crop for a longer time. In commercial production, broilers are normally exposed to 22-24 h of continuous light. Changing from continuous to intermittent light can change the bird's eating pattern from nibbling to meal feeding. This would be expected to result in feed residing in the crop for a longer time. Studies showed that the crop content of birds given intermittent lighting, *i.e.*, 1L:3D, is three times more than that of chicks on continuous light at the end of the lighting period (Hooppaw and Goodman, 1976). Therefore, changing light pattern from continuous light to intermittent light may enhance the efficacy of exogenous enzymes through prolonging the residing time of diet in the crop.

Objective

The objective of these studies was to investigate the effects of supplementation of α-galactosidase, amylase and acidification of the diet, and water acidification, alone or in combination on nutrient digestion and growth performance of broiler chicks fed a corn-soybean meal-based diet.

Materials and Methods

Animals and Housing

Four experiments were conducted at the UK Poultry Research Facility. Each experiment used male chicks of a female broiler breeder line obtained from Avian Division, Cobb-Vantress, Monticello, KY. Chicks were 1 d of age at the start of each experiment. The chicks were housed in mesh wire-floored pullet starter cages (61 cm x 51 cm x 36 cm) in an environmentally controlled room. All experiments used a randomized complete block design, with blocks based on physical location of the cages within the room. Either continuous light (22L:2D) or six cycles of intermittent light (1L:3D) was provided daily. Each cage had one feeder that was removable for weighing and two nipple adjustable drinkers. Feed and water were supplied on an *ad libitium* basis. The temperature in the room was 31°C for the first week and then lowered to 27°C for the remainder of the study. Thick papers were placed on the floor of the cages for the first week. Daily animal care sheets were filled out providing a record of mortality, temperature, and feed and water availability. Bird mortality was monitored daily. If a bird died within the first 4 days, it was replaced by a bird of similar weight from a corresponding extra cage receiving the same diet. Birds and feed were weighed initially and then weekly. After each week, mortality, average body weight gain, average daily feed intake and gain to feed ratio were calculated. Chromic oxide (Cr_2O_3) was included and replaced the corn in all the experimental diets at 0.25% as an internal marker for the measurement of the digestibility. The study protocol was approved by the University of Kentucky Institutional Animal Care and Use Committee (IACUC).

Materials

Commercial fungal sources of α-galactosidase and amylase were supplied by Alltech Inc., Nicholasville, KY. The measured activity of α-galactosidase was 1724 GalU/g and amylase was 9705 FAU/g. Food grade anhydrous citric acid was purchased from Roche Vitamins Inc., Parsippany, NJ. Chromic oxide (Cr_2O_3) (C333-3) was purchased from Fisher Scientific Inc., Pittsburgh, PA. ACID-PAK 4-WAY was supplied by Alltech Inc., Nicholasville, KY. ACID-PAK 4-WAY is an organic acidifier for drinking water. It is a mixture of organic acids, salts and fermentation extracts. The major ingredients contained in this product include water, citric acid, sodium citrate, acetic acid, sodium chloride, potassium chloride, zinc sulfate, iron sulfate, magnesium sulfate, ethyl vanillin, dried *Aspergillus niger* fermentation extract and dried *Bacillus subtilis* fermentation extract. Also, this acidifier contains amylase and protease. The

recommended activity of amylase is 95,000 BAU/gallon and of protease is 159,000 HUT/gallon. Other feed ingredients were supplied by the Feed Mill at the University of Kentucky.

Experimental Basal Diets

Low energy and normal energy corn-soybean meal basal diets were used in these studies. The low energy basal diet was formulated to contain approximately 88% of the energy level in the normal energy basal diet (2.74 vs. 3.11 Mcal/kg). The latter diet contained an energy level that was similar to that recommended for commercial production (Classic and HI-Y Broiler Management Guide, HUBBARD farms, 2004). The level of other nutrients met or exceeded NRC (1994) recommendations. The ingredient and calculated composition of nutrients are presented in Table 6.1 and Table 6.2, respectively.

Table 6.1. Ingredient composition of the basal diet

Ingredients	Normal energy basal	Low energy basal
	(%)	
Corn	53.55	52.00
Soybean meal (48%CP)	38.50	36.00
Alfalfa (17%CP)	–	8.40
Corn oil	4.00	–
Salt	0.45	0.44
Limestone	1.32	1.06
Dicalcium phosphate	1.75	1.68
DL-Methionine	0.18	0.17
Vitamin-mineral mix[1]	0.25	0.25

[1]Supplied per kg diet: 11,025 I.U. vitamin A, 3,528 I.U. vitamin D_3, 33 I.U. vitamin E, 0.91 mg vitamin K, 2 mg thiamin, 8 mg riboflavin, 55 mg niacin, 18 mg Ca pantothenate, 5 mg vitamin B-6, 0.221 mg biotin, 1 mg folic acid, 478 mg choline, 28 μg vitamin B-12, 75 mg zinc, 40 mg iron, 64 mg manganese, 10 mg copper, 2 mg iodine and 0.3 mg selenium.

Table 6.2. Nutrient composition of the basal diet [1]

Nutrient	Normal energy basal	Low energy basal
AME_n, Mcal/kg	3.11	2.74
CP, %	22.87	22.97
Fat, %	6.21	2.34
Fiber, %	2.63	4.52
Ca, %	1.00	1.00
Available P, %	0.45	0.45
Methionine, %	0.54	0.53
Methionine + cystine, %	0.90	0.89
Lysine, %	1.30	1.28
Sodium, %	0.20	0.20

[1]Values reported are calculated and “as-is” basis.

Experimental Design and Dietary Treatments

Experiment 4.1

This experiment was conducted to study the effects of simultaneous application of α-galactosidase and citric acid on nutrient digestibility and growth performance of broiler chicks. One hundred forty-four broiler chicks were used in this experiment. Each experimental unit consisted of one cage of six birds. There were six replicates in a treatment with four dietary treatments. Dietary treatments were randomly distributed to cages within each of three blocks.

The low energy corn-soybean meal basal diet was used (Table 6.1 and 6.2). Four dietary treatments consisted of feeding a basal diet alone, or supplemented with 2% citric acid, 0.1% (1724 units/kg) α-galactosidase, or both. Alpha-galactosidase and citric acid replaced the corn in the basal diet. Continuous light (22L:2D) was provided daily. The experiment lasted three weeks. On Day 7 and Day 14, excreta samples were collected from each cage for 24 h. The collection was conducted by hanging the collection board underneath the cage. Then excreta samples were dried in an oven at 60°C for 72 hours.

Experiment 4.2

Experiment 4.2 was designed to study the effects of simultaneous supplementation of α-galactosidase, amylase and citric acid on nutrient digestibility and growth performance of broiler chicks. One hundred forty-four chicks were used in this experiment. Each experimental unit consisted of one cage of six chicks. There were six replicates in a treatment with four dietary treatments. Dietary treatments were randomly distributed to cages within each of three blocks.

In this experiment, the low energy basal diet (Tables 6.1 and 6.2) was fed alone, or supplemented with 0.2% enzyme cocktail (1724 units/kg α-galactosidase and 9705 units/kg amylase), 1% citric acid, or 0.2% enzyme cocktail plus 1% citric acid. An intermittent lighting regime supplied six cycles of 1L:3D daily. The experiment lasted three weeks. On Day 18, excreta samples were collected and dried from each cage as described previously.

Experiment 4.3

The objective of this experiment was to investigate the effects of dietary α-galactosidase and acidification of the diet and the water on nutrient digestibility and growth performance of broiler chicks. Two hundred eighty-eight chicks were used in this experiment. Each experimental unit consisted of one cage of six birds. Six replicates were assigned to each of eight dietary treatments. Dietary treatments were randomly distributed to cages within each of six blocks.

The low energy basal diet (Tables 6.1 and 6.2) was used in this experiment. Treatment structure consisted of a 2 x 2 x 2 factorial arrangement. Three factors were: two dietary levels of citric acid (0 or 0.5%), two dietary levels of α-galactosidase (0 or 0.4%) and two water sources (normal or acidified water). Water was acidified by ACID-PAK 4-WAY at 2 ml/L water. Water was weighed into a big container. ACID-PAK 4-WAY was added in the water based on the recommended concentration. Then the water was thoroughly mixed. The measured pH of acidified water was 3.26. Jar drinker (500 ml) was used to supply water for the chicks. Water consumption was recorded daily. The eight treatments are listed in Table 6.3. Alpha-galactosidase and citric acid replaced the corn in the basal diet. An intermittent lighting regime provided six cycles of 1L:3D daily. The experiment lasted two weeks. On Day 14, excreta samples were collected and dried from each cage as described previously.

Table 6.3. Experimental treatments (Experiment 4.3)

Treatment	Diet
1	Low energy basal (B), normal water
2	B, acidified water
3	B + 0.5% citric acid, normal water
4	B + 0.5% citric acid, acidified water
5	B + 0.4% α-galactosidase*, normal water
6	B + 0.4% α-galactosidase, acidified water
7	B + 0.4% α-galactosidase + 0.5% citric acid, normal water
8	B + 0.4% α-galactosidase + 0.5% citric acid, acidified water

*Equivalent to 6896 units/kg diet.

Experiment 4.4

The purpose of this experiment was to study the effects of simultaneous application of citric acid and α-galactosidase on nutrient digestibility and growth performance of broilers fed corn-soy basal diets with different energy levels. Five hundred seventy-six chicks were used in this experiment. Each experimental unit consisted of two cages of twelve birds (one cage on the top tier of the battery and one cage on the bottom tier directly below). There were six replicates in a treatment with eight dietary treatments. Dietary treatments were randomly distributed to cages within each of six blocks.

The normal energy and low energy basal diets shown in Table 6.1 and 6.2 were used in this study. Treatment structure consisted of a 2 x 2 x 2 factorial arrangement. The three dietary factors were: two levels of energy (low or normal), two levels of citric acid (0 or 1.5%) and two levels of α-galactosidase (0 or 0.1%). The eight dietary treatments are listed in Table 6.4. Alpha-galactosidase and citric acid replaced the corn in the basal diet. Continuous light (22L:2D) was provided daily. The experiment lasted three weeks. On Day 18, excreta samples were collected from each cage for 24 h and dried with the methods described previously. On Day 22, six birds from each treatment (a total of 48 birds) were randomly selected and not given feed for 12 h. Then, the birds were fed for 20 min before the feeders were removed. Thirty minutes after the removal of the feeder, three birds from each treatment (a total of 24 birds) were killed by asphyxiation with argon gas followed by cervical dislocation. Digesta from the crop was

collected. The digesta pH was determined immediately after collection. Another sample was obtained from three more birds per treatment 60 min after removal of the feeder. The crop digesta was kept on ice until subsequent storage at -10°C for the measurement of reducing sugars.

Table 6.4. Dietary treatments (Experiment 4.4)

Treatment	Diet
1	Low energy basal diet (LB)
2	LB + 0.1% α-galactosidase*
3	LB + 1.5% citric acid
4	LB + 0.1% α-galactosidase + 1.5% citric acid
5	Normal energy basal diet (NB)
6	NB + 0.1% α-galactosidase
7	NB + 1.5% citric acid
8	NB + 0.1% α-galactosidase + 1.5% citric acid

*Equivalent to 1724 units/kg diet.

Laboratory Analyses

Samples of feed and oven-dried excreta were ground in a coffee grinder to a fine texture. Dry matter (DM) was analyzed using the method from the AOAC (1995), where DM was determined after drying the samples overnight in an oven at 105°C, and moisture content was calculated by difference. Gross energy (GE) was determined by measuring the heat of combustion in the samples using a Parr 1281 Oxygen Bomb Calorimeter (Appendix 11). Nitrogen content was assayed using the Dumas methodology in a LECO FP-2000 Automated Analyzer (AOAC 990.03) (Appendix 9). The determination of neutral detergent fiber (NDF) was based on the modified assay from Van Soest (1967) (Appendix 12). Chromium content in the samples (from Cr_2O_3) was analyzed using a procedure established by Williams *et al.* (1962) (Appendix 10). The pH of the crop content was measured with the method described by Radcliffe *et al.* (1998). A procedure proposed by Miller *et al.* (1959) was used to determine the reducing sugar content in the crop digesta.

Statistical Analysis

Data were subjected to ANOVA for a randomized complete block design using linear models of Statistix V.8 (2003) (Analytical Software, Tallahassee, FL). Mean differences were determined using Fisher's least significant difference test. Significance was declared when probability was less than 5%. Orthogonal contrasts (Snedector and Cochran, 1989) were used in Experiment 4.4 to identify the effects of α-galactosidase, citric acid or both on the pH and reducing sugar concentration in the crop content. The coefficients for the contrasts are outlined in Table 6.5.

Table 6.5. Coefficients for treatment orthogonal contrasts (Experiment 4.4)

Contrast description	Dietary treatment coefficient							
	1	2	3	4	5	6	7	8
	Low basal (LB)	LB + enzyme	LB + citrate	LB + enzyme + citrate	Normal basal (NB)	NB + enzyme	NB + citrate	NB + enzyme + citrate
Enzyme supplementation: no vs. + 0.1% (1, 3, 5 & 7 vs. 2, 4, 6 & 8)	1	-1	1	-1	1	-1	1	-1
Acid supplementation: no vs. + 1.5% (1, 2, 5 & 6 vs. 3, 4, 7 & 8)	1	1	-1	-1	1	1	-1	-1
Energy level: low vs. high (1, 2, 3 & 4 vs. 5, 6, 7 & 8)	1	1	1	1	-1	-1	-1	-1
Enzyme vs. enzyme + acid (2 & 6 vs. 4 & 8)	0	1	0	-1	0	1	0	-1
Acid vs. acid + enzyme (3 & 7 vs. 4 & 8)	0	0	1	-1	0	0	1	-1

Results

Experiment 4.1

The effects of α-galactosidase and citric acid on body weight gain, feed intake and gain to feed ratio of broiler chicks measured at Day 7, Day 14 and Day 21 are listed in Table 6.6. The chicks fed 0.1% α-galactosidase consumed more feed ($P<0.01$) and gained more body weight ($P<0.05$) during the overall 21-day experimental period. Citric acid supplementation depressed ($P< 0.01$) overall weight gain and feed intake. In the first two weeks, there was a significant enzyme by citric acid interaction. The depression of weight gain and gain to feed ratio caused by the use of citric acid was corrected ($P<0.01$) by the inclusion of α-galactosidase. However, for the entire 21-day period, this interaction was not significant.

The effects of dietary α-galactosidase and citric acid on AME_n of the diets and the digestibility of DM, CP and NDF measured at Day 7 and Day 14 are shown in Table 6.7. Alpha-galactosidase increased AME_n ($P<0.05$) of the diets and the digestibility of CP ($P<0.05$) and NDF ($P<0.01$). Citric acid increased the digestibility of DM ($P<0.01$), CP ($P<0.05$) and NDF ($P<0.01$). The simultaneous supplementation of α-galactosidase and citric acid further increased AME_n ($P<0.05$) of the diets and the digestibility of DM ($P<0.05$) and NDF ($P<0.01$).

Experiment 4.2

The results of this experiment are presented in Table 6.8 and Table 6.9. The addition of enzymes did not significantly affect growth performance (Table 6.8) or digestibility (Table 6.9). Citric acid decreased ($P<0.05$) weight gain and feed intake during first two weeks of the trail. A significant interaction ($P<0.05$) between enzymes and citric acid on the digestibility of CP was observed. The digestibility of CP was significantly lower for the group fed enzyme plus citric acid compared with the group fed enzyme alone.

Table 6.6. Effects of α-galactosidase and citric aid on growth performance of broiler chicks (Experiment 4.1)[1]

Treatment		Weight gain (g/bird)			Feed intake (kg/bird)			Gain to feed ratio (g/g)		
		1 – 7 d	1 – 14 d	1 – 21 d	1 – 7 d	1 – 14 d	1 – 21 d	1 – 7 d	1 – 14 d	1 – 21 d
Enzyme, %	Citrate, %									
0		104	344	714	200	524	1123	0.52	0.65	0.63
0.1		104	361	766	198	537	1164	0.53	0.67	0.66
	0	108	369	775	201	545	1173	0.54	0.68	0.66
	2	100	336	706	197	515	1114	0.51	0.65	0.63
0	0	112[a]	376[a]	769	198	542	1166	0.57[a]	0.69[a]	0.66
0	2	95[b]	313[b]	659	203	505	1081	0.47[b]	0.62[b]	0.61
0.1	0	104[ab]	362[a]	780	204	548	1180	0.51[ab]	0.66[a]	0.66
0.1	2	105[ab]	359[a]	753	192	526	1147	0.55[ab]	0.68[a]	0.66
EMS[2]		101	633	3164	103	315	918	0.005	0.001	0.002
Source of variation		Significance of treatment effect[3]								
Enzyme		ns	ns	*	ns	ns	**	ns	ns	ns
Acid		ns	**	**	ns	**	**	ns	ns	ns
Enzyme x acid		*	**	ns	ns	ns	ns	*	**	ns

[1]Six replicate groups of six birds were assigned to each of four treatments.
[2]Error mean square.
[3]Significance of treatment effect: ns = not significant; * = P<0.05; ** = P<0.01.
[a, b] Means within a column that lack a common superscript letter differ.

Table 6.7. Effects of α-galactosidase and citric aid on AME_n and digestibility of DM, CP and NDF (Experiment 4.1)[1]

Treatment		DM, %		CP, %		NDF, %		AME_n[2], Kcal/kg	
		d 7	d 14	d 7	d 14	d 7	d 14	d 7	d 14
Enzyme, %	Citrate, %								
0		68.8	67.6	63.1	62.2	16.8	13.5	3128	3070
0.1		69.3	68.2	64.8	64.3	22.0	18.3	3157	3122
	0	69.4	67.0	65.6	62.2	18.1	12.6	3174	3071
	2	68.8	68.8	62.3	64.4	20.7	19.2	3111	3120
0	0	69.8	67.2[a]	66.9	61.5	16.7	11.9[a]	3202[a]	3072[a]
0	2	67.9	68.1[a]	59.3	62.8	17.0	15.1[b]	3055[b]	3067[a]
0.1	0	69.0	66.9[a]	64.4	62.8	19.6	13.3[ab]	3147[ab]	3070[a]
0.1	2	69.5	69.6[b]	65.3	65.9	24.4	23.3[c]	3167[a]	3174[b]
EMS[3]		2.64	1.26	17.9	3.66	10.1	3.92	4913	2201
Source of variation		Significance of treatment effect[4]							
Enzyme		ns	ns	ns	*	**	**	ns	*
Acid		ns	**	ns	*	ns	**	ns	ns
Enzyme x acid		ns	*	ns	ns	ns	**	*	*

[1]Six replicate groups of six birds were assigned to each of four treatments.
[2]Data presented are dry matter basis.
[3]Error mean square.
[4]Significance of treatment effect: ns = not significant; * = $P<0.05$; ** = $P<0.01$.
[a, b] Means within a column that lack a common superscript letter differ.

Table 6.8. Effects of α-galactosidase, amylase and citric aid on growth performance of broiler chicks (Experiment 4.2)[1]

Treatment		Weight gain (g/bird)			Feed intake (kg/bird)			Gain to feed ratio (g/g)		
		1 – 7 d	1 – 14 d	1 – 21 d	1 – 7 d	1 – 14 d	1 – 21 d	1 – 7 d	1 – 14 d	1 – 21 d
Enzyme, %	Citrate, %									
0		95	334	721	146	453	1029	0.65	0.74	0.70
0.2		97	337	736	148	457	1037	0.66	0.74	0.71
	0	99	344	738	151	464	1045	0.66	0.74	0.71
	1	93	327	719	143	445	1020	0.65	0.73	0.70
0	0	99	343	725	149	463	1030	0.67	0.74	0.70
0	1	90	325	716	143	443	1027	0.63	0.73	0.70
0.2	0	98	345	750	153	466	1060	0.65	0.74	0.71
0.2	1	95	329	721	143	447	1013	0.66	0.74	0.71
EMS[2]		48	315	1537	52	334	1444	0.002	0.0004	0.0004
Source of variation		Significance of treatment effect[3]								
Enzyme		ns	ns	ns	ns	ns	ns	ns	ns	ns
Acid		*	*	ns	*	*	ns	ns	ns	ns
Enzyme x acid		ns	ns	ns	ns	ns	ns	ns	ns	ns

[1]Six replicate groups of six birds were assigned to each of four treatments.
[2]Error mean square.
[3]Significance of treatment effect: ns = not significant; * = $P<0.05$.

Table 6.9. Effects of α-galactosidase, amylase and citric acid on AME_n and digestibility of DM and CP (Experiment 4.2)[1]

Treatment		DM, %	CP, %	AME_n[2], Kcal/kg
Enzyme, %	Citrate, %			
0		67.3	60.0	3026
0.2		67.1	60.1	3015
	0	67.5	60.8	3029
	1	66.9	59.4	3012
0	0	67.2	59.1[ab]	3027
0	1	67.4	60.9[ab]	3026
0.2	0	67.8	62.4[a]	3032
0.2	1	66.5	57.9[b]	2999
EMS[3]		1.52	8.07	2799
Source of variation		——— Significance of treatment effect[4] ———		
Enzyme		ns	ns	ns
Acid		ns	ns	ns
Enzyme x acid		ns	*	ns

[1]Six replicate groups of six birds were assigned to each of four treatments.
[2]Data presented are dry matter basis.
[3]Error mean square.
[4]Significance of treatment effect: ns = not significant; * = P<0.05.
[a, b] Means within a column that lack a common superscript letter differ.

Experiment 4.3

The effects of dietary α-galactosidase and citric acid and water acidification on chick performance, water consumption, AME_n and digestibility of DM and CP are presented in Table 6.10 through 6.14. There were no effects of dietary α-galactosidase or water acidification on any variable (Tables 6.10 and 6.11). Dietary supplementation with citric acid depressed feed intake during the first week (P<0.05) and AME_n of the diet (P<0.01) (Table 6.12). A significant interaction (P<0.01) of α-galactosidase and citric acid on weight gain and gain to feed ratio was

found (Table 6.10). The birds fed citric acid or α-galactosidase alone gained less body weight than the birds fed the basal diet alone or the basal plus both citric acid and α-galactosidase (Table 6.13). A similar effect was observed on the gain to feed ratio (Table 6.13). Chicks fed both citric acid and acidified water had lower digestibility of DM ($P<0.05$) and CP ($P<0.01$) than those fed citric acid or acidified water alone resulting in a significant interaction (Table 6.11 and 6.14).

Experiment 4.4

The results of this experiment are presented in Table 6.15 through Table 6.22. Dietary citric acid significantly decreased ($P<0.01$) the pH of the crop content (Table 6.15) (6.2 vs. 5.5). There were large differences in reducing sugar concentration of crop contents among dietary treatments (Table 6.15). The contrasts shown in Table 6.15 indicated that crop contents from chicks fed α-galactosidase contained more ($P<0.01$) reducing sugars than did crop contents from chicks fed no α-galactosidase (8.0 vs. 12.5). Crop contents from chicks fed citric acid contained more ($P<0.01$) reducing sugars than did crop contents from chicks fed no citric acid (7.9 vs. 12.5). Crop contents from chicks fed both α-galactosidase and citric acid contained more ($P<0.05$) reducing sugars than did crop contents from chicks fed α-galactosidase or citric acid alone (9.7 vs. 15.3).

Birds fed the lower energy diet were lighter in weight, ate more feed, and had lower gain to feed ratio, AME_n and DM digestibility than the birds given the normal energy diet (Table 6.16, 6.17 and 6.18). Dietary inclusion of α-galactosidase significantly ($P<0.01$) improved the AME_n of the diets, although no effect on growth performance was noticed (Table 6.16, 6.17 and 6.18). Citric acid decreased ($P<0.01$) the AME_n of the diet (Table 6.17 and 6.18). Chicks fed citric acid had lower ($P<0.05$) gain to feed ratio than did the chicks fed both α-galactosidase and citric acid (Table 6.19). The inclusion of α-galactosidase in the low energy basal diets caused an 11.6% improvement ($P<0.01$) of AME_n, while the addition of α-galactosidase to the normal basal diet had no effect on the AME_n of the diets (Table 6.20). The reduction of AME_n due to the supplementation of citric acid occurred only when citric acid was added to diets without α-galactosidase or to the low energy diets (Table 6.21 and 6.22).

Table 6.10. Effects of dietary α-galactosidase (E) and citric acid (CA) and water acidification (WA) on growth performance of broiler chicks (Experiment 4.3)[1]

Treatment	Factors			Cumulative weight gain (g/bird)		Cumulative feed intake (g/bird)		Cumulative Gain to feed ratio (g/g)	
	E	CA	WA	7d	14d	7d	14d	7d	14d
1	-	-	-	112	378	167	549	0.68	0.69
2	-	-	+	105	368	166	536	0.63	0.69
3	-	+	-	101	356	161	522	0.63	0.68
4	-	+	+	101	343	160	523	0.63	0.65
5	+	-	-	107	342	170	530	0.63	0.65
6	+	-	+	102	340	179	533	0.57	0.64
7	+	+	-	102	359	163	523	0.63	0.69
8	+	+	+	103	361	164	538	0.63	0.67
SEM[2]				3.78	9.18	4.79	8.37	0.03	0.01
Source of variation				——— Significance of treatment effect[3] ———					
E				ns	ns	ns	ns	ns	ns
CA				ns	ns	*	ns	ns	ns
WA				ns	ns	ns	ns	ns	ns
E x CA				ns	**	ns	ns	ns	**
E x WA				ns	ns	ns	ns	ns	ns
CA x WA				ns	ns	ns	ns	ns	ns
E x CA x WA				ns	ns	ns	ns	ns	ns

[1]Data presented are means from six groups of six birds.
[2]Sdandard error of the mean.
[3]Significance of treatment effect: ns = not significant; * = P<0.05; ** = P<0.01.

Table 6.11. Effects of dietary α-galactosidase (E) and citric acid (CA) and water acidification (WA) on water consumption, AME_n and digestibility of DM and CP (Experiment 4.3)[1]

Treatment	Factors			Water consumption (kg/bird/d)	DM digestibility (%)	CP digestibility (%)	AME_n[2] (Kcal/kg)
	E	CA	WA				
1	-	-	-	1.32	65.8	59.3	3026
2	-	-	+	1.30	67.1	62.0	3079
3	-	+	-	1.26	66.5	62.4	3014
4	-	+	+	1.28	65.7	59.3	2979
5	+	-	-	1.30	66.8	61.3	3069
6	+	-	+	1.25	66.5	60.8	3039
7	+	+	-	1.22	67.1	62.5	3036
8	+	+	+	1.25	65.6	59.5	2987
SEM[3]				0.03	0.53	0.83	24.8
Source of variation				Significance of treatment effect[4]			
E				ns	ns	ns	ns
CA				ns	ns	ns	**
WA				ns	ns	ns	ns
E x CA				ns	ns	ns	ns
E x WA				ns	ns	ns	ns
CA x WA				ns	*	**	ns
E x CA x WA				ns	ns	ns	ns

[1]Data presented are means from six groups of six birds.
[2]Data presented are dry matter basis.
[3]Sdandard error of the mean.
[4]Significance of treatment effect: ns = not significant; * = P<0.05; ** = P<0.01.

Table 6.12. Least squares means for the main effects of α-galactosidase, citric acid and ACID-PAK 4-WAY on growth performance, water consumption, AME_n and digestibility of DM and CP (Experiment 4.3)[1]

Parameter	α-Galactosidase		Citric acid		ACID-PAK 4-WAY		SEM[2]
	no	+ 0.4%	no	+ 0.5%	no	+ 2 ml/L	
Day 1 – 7							
Weight gain, g/bird	105	104	106	102	105	103	1.89
Feed intake, g/bird	163	169	171[a]	162[b]	165	167	2.39
Gain to feed ratio, g/g	0.64	0.62	0.63	0.63	0.64	0.62	0.01
Day 1 - 14							
Weight gain, g/bird	361	350	357	354	359	353	4.59
Feed intake, g/bird	532	531	537	526	531	533	4.19
Gain to feed ratio, g/g	0.68	0.66	0.66	0.67	0.68	0.66	0.01
Water consumption, kg/bird	1.29	1.25	1.29	1.25	1.27	1.27	0.02
AME_n[3], Kcal/kg	3024	3032	3053[a]	3004[b]	3036	3021	12.4
DM digestibility, %	66.3	66.5	66.6	66.2	66.5	66.2	0.26
CP digestibility, %	60.7	61.0	60.8	60.9	61.3	60.4	0.42

[1]Data presented are means from twenty four groups of six birds.
[2]Sdandard error of the mean.
[3]Data presented are dry matter basis.
[a, b] Means within a row that lack a common superscript letter differ, P<0.05.

Table 6.13. Interactive effect of α-galactosidase and citric acid on overall growth performance (14 days) of broiler chicks (Experiment 4.3)[1]

α-Galactosidase	Citric acid	Weight gain (g/bird)	Feed intake (g/bird)	Gain to feed ratio (g/g)
-	-	373[a]	542	0.69[a]
-	+	349[b]	522	0.67[ab]
+	-	341[b]	531	0.64[b]
+	+	360[ab]	531	0.68[a]
SEM[2]		6.49	5.92	0.009

[1]Data presented are means from twelve groups of six birds.
[2]Sdandard error of the mean.
[a, b] Means within a column that lack a common superscript letter differ, P<0.01.

Table 6.14. Interactive effect of dietary citric acid and water acidification on AME_n and digestibility of DM and CP (Experiment 4.3)[1]

Citric acid	ACID-PAK 4-WAY	DM digestibility (%)	CP digestibility (%)	AME_n[2] (Kcal/kg)
-	-	66.3[ab]	60.2[bc]	3047
-	+	66.8[a]	61.4[ab]	3059
+	-	66.8[a]	62.4[a]	3025
+	+	65.6[b]	59.4[c]	2983
SEM[3]		0.37	0.59	17.5

[1]Data presented are means from twelve groups of six birds.
[2]Data presented are dry matter basis.
[3]Sdandard error of the mean.
[a, b, c] Means within a column that lack a common superscript letter differ, P<0.05.

Table 6.15. Effects of dietary α-galactosidase (E) and citric acid (CA) on pH and reducing sugar concentration in crop content (Experiment 4.4)[1]

Diet		pH	Reducing sugar[2], g/kg
1	Low energy basal (LB)	6.2	6.4
2	LB + E	6.2	10.6
3	LB + CA	5.6	9.1
4	LB + E + CA	5.4	15.1
5	Normal energy basal (NB)	6.2	5.9
6	NB + E	6.2	8.8
7	NB + CA	5.5	10.5
8	NB + E + CA	5.2	15.4
	SEM[3]	0.11	1.47
	Orthogonal contrasts[4]		
Enzyme: no vs. + 0.1% (1, 3, 5 & 7 vs. 2, 4, 6 & 8)		ns	**
Acid: no vs. + 1.5% (1, 2, 5 & 6 vs. 3, 4, 7& 8)		**	**
Energy: low vs. normal (1, 2, 3 & 4 vs. 5, 6, 7 & 8)		ns	ns
Enzyme vs. Enzyme + acid (2 & 6 vs. 4 & 8)		**	*
Acid vs. Acid + enzyme (3 & 7 vs. 4 & 8)		ns	*

[1]Data presented are means of duplicates of three samples.
[2]Expressed as g per kg air-dried crop content.
[3]Sdandard error of the mean.
[4]Significance of contrast: ns = not significant, * = P < 0.05, ** = P < 0.01.

Table 6.16. Effects of dietary energy level (EL), α-galactosidase (E) and citric acid (CA) on growth performance of broiler chicks (Experiment 4.4)[1]

Treatment	Factors			Weight gain, g/bird			Feed intake, g/bird			Gain to feed ratio, g/g		
	EL	E	CA	1 - 7	1 - 14	1 - 21	1 - 7	1 - 14	1 - 21	1 - 7	1 - 14	1 - 21
1	Low	-	-	99.0	356	764	150	522	1128	0.68	0.68	0.68
2	Low	-	+	98.2	344	750	148	507	1113	0.67	0.68	0.67
3	Low	+	-	95.2	340	745	148	515	1117	0.65	0.66	0.67
4	Low	+	+	94.9	342	748	142	497	1091	0.67	0.69	0.69
5	Normal	-	-	98.7	362	827	142	491	1080	0.70	0.74	0.77
6	Normal	-	+	98.6	362	800	143	496	1086	0.69	0.73	0.74
7	Normal	+	-	97.2	370	833	140	498	1099	0.69	0.74	0.76
8	Normal	+	+	104	380	832	143	504	1094	0.73	0.75	0.76
	SEM[2]			2.67	7.53	14.4	1.82	6.77	13.9	0.013	0.013	0.008
Source of variation				Significance of treatment effect[3]								
EL				ns	**	**	**	*	*	**	**	**
E				ns	ns	ns	ns	ns	ns	ns	ns	ns
CA				ns	ns	ns	ns	ns	ns	ns	ns	ns
EL x E				ns	ns	ns	ns	ns	ns	ns	ns	ns
EL x CA				ns	ns	ns	ns	*	ns	ns	ns	ns
E x CA				ns	ns	ns	ns	ns	ns	ns	ns	*
EL x E x CA				ns	ns	ns	ns	ns	ns	ns	ns	ns

[1]Data presented are means from six groups of twelve birds.
[2]Sdandard error of the mean.
[3]Significance of treatment effect: ns = not significant; * = P<0.05; ** = P<0.01.

Table 6.17. Effects of dietary energy level (EL), α-galactosidase (E) and citric acid (CA) on AME_n and digestibility of DM and CP (Experiment 4.4)[1]

Treatment	Factors			DM digestibility (%)	CP digestibility (%)	AME_n[2] (Kcal/kg)
	EL	E	CA			
1	Low	-	-	66.1	58.6	2992[b]
2	Low	-	+	67.0	60.7	2493[c]
3	Low	+	-	66.8	61.0	3017[b]
4	Low	+	+	69.3	61.7	3105[b]
5	Normal	-	-	72.2	64.1	3448[a]
6	Normal	-	+	72.0	63.2	3436[a]
7	Normal	+	-	72.9	64.2	3471[a]
8	Normal	+	+	71.4	62.1	3415[a]
SEM[3]				1.23	2.41	51.9
Source of variation				— Significance of treatment effect[4] —		
EL				**	ns	**
E				ns	ns	**
CA				ns	ns	**
EL x E				ns	ns	**
EL x CA				ns	ns	*
E x CA				ns	ns	**
EL x E x CA				ns	ns	**

[1]Data presented are means from six groups of twelve birds.
[2]Data presented are dry matter basis.
[3]Sdandard error of the mean.
[4]Significance of treatment effect: ns = not significant; * = P<0.05; ** = P<0.01.
[a, b, c] means within a column that lack a common superscript letter differ.

Table 6.18. Least squares means for the main effects of energy level, α-galactosidase and citric acid on growth performance, AME_n and digestibility of DM and CP (Experiment 4.4)[1]

Parameter	Energy level, Kcal/kg		α-Galactosidase, %		Citric acid, %		SEM[2]
	2736	3107	0	0.1	0	1.5	
Day 1 - 7							
Weight gain, g/bird	97	100	99	98	98	99	1.33
Feed intake, g/bird	147[a]	142[b]	146	143	145	144	0.91
Gain to feed ratio, g/g	0.67[a]	0.70[b]	0.68	0.68	0.68	0.69	0.007
Day 1 - 14							
Weight gain, g/bird	345[a]	368[b]	356	358	357	357	3.77
Feed intake, g/bird	510[a]	497[b]	504	503	506	501	3.38
Gain to feed ratio, g/g	0.68[a]	0.74[b]	0.71	0.71	0.71	0.71	0.007
Day 1 - 21							
Weight gain, g/bird	712[a]	823[b]	785	789	792	782	7.21
Feed intake, g/bird	1112[a]	1090[b]	1102	1101	1106	1096	6.97
Gain to feed ratio, g/g	0.68[a]	0.75[b]	0.71	0.72	0.72	0.71	0.004
AME_n[2], Kcal/kg	2902[a]	3442[b]	3092[c]	3252[d]	3232[e]	3113[f]	25.9
DM digestibility, %	67.3[a]	72.1[b]	69.3	70.1	69.5	69.9	0.61
CP digestibility, %	60.5	63.4	61.7	62.3	62.0	61.9	1.20

[1]Data presented are means from twenty four groups of twelve birds.
[2]Sdandard error of the mean.
[3]Data presented are dry matter basis.
[a, b, c, d, e, f] means within a row that lack a common superscript letter differ, P<0.05 or 0.01.

Table 6.19. Interactive effect of dietary α-galactosidase and citric acid on overall growth performance (Experiment 4.4)[1]

α-Galactosidase	Citric acid	Weight gain, g/bird	Feed intake, g/bird	Gain to feed ratio, g/g
-	-	796	1104	0.721[ab]
-	+	775	1100	0.705[b]
+	-	789	1108	0.712[ab]
+	+	790	1093	0.723[a]
SEM[2]		10.2	9.9	0.006

[1]Data presented are means from twelve groups of twelve birds.
[2]Sdandard error of the mean.
[a, b] means within a column that lack a common superscript letter differ, P<0.05.

Table 6.20. Interactive effect of dietary energy level and α-galactosidase on AME_n and digestibility of DM and CP (Experiment 4.4)[1]

Energy level	α-Galactosidase	DM digestibility (%)	CP digestibility (%)	AME_n[2] (Kcal/kg)
Low	-	66.5	59.7	2743[c]
Low	+	68.1	61.3	3061[b]
Normal	-	72.1	63.7	3442[a]
Normal	+	72.1	63.2	3443[a]
SEM[3]		0.87	1.70	36.7

[1]Data presented are means from twelve groups of twelve birds.
[2]Data presented are dry matter basis.
[3]Sdandard error of the mean.
[a, b, c] means within a column that lack a common superscript letter differ, P<0.01.

Table 6.21. Interactive effect of dietary α-galactosidase and citric acid on AME_n and digestibility of DM and CP (Experiment 4.4)[1]

α-Galactosidase	Citric acid	DM digestibility (%)	CP digestibility (%)	AME_n[2] (Kcal/kg)
-	-	69.2	61.4	3220[a]
-	+	69.5	62.0	2965[b]
+	-	69.8	62.6	3244[a]
+	+	70.3	61.9	3260[a]
SEM[3]		0.87	1.70	36.7

[1]Data presented are means from twelve groups of twelve birds.
[2]Data presented are dry matter basis.
[3]Sdandard error of the mean.
[a, b] means within a column that lack a common superscript letter differ, P<0.01.

Table 6.22. Interactive effect of dietary energy level and citric acid on AME_n and digestibility of DM and CP (Experiment 4.4)[1]

Energy level	Citric acid	DM digestibility (%)	CP digestibility (%)	AME_n[2] (Kcal/kg)
Low	-	66.5	59.8	3005[c]
Low	+	68.2	61.2	2799[b]
Normal	-	72.5	64.2	3459[a]
Normal	+	71.7	62.7	3426[a]
SEM[3]		0.61	1.70	36.7

[1]Data presented are means from twelve groups of twelve birds.
[2]Data presented are dry matter basis.
[3]Sdandard error of the mean.
[a, b, c] means within a column that lack a common superscript letter differ, P<0.01.

Discussion

Effects of α-Galactosidase on Growth Performance, AME_n and Digestibility of DM, CP and NDF

Experiment 4.1 showed an increase of weight gain and feed intake by supplementation of α-galactosidase. This result was not repeated in later studies. Dietary α-galactosidase increased the digestibility of NDF and reducing sugar concentration in crop contents meaning hydrolysis of α-galactosides by α-galactosidase occurred. Improved digestibility of CP by α-galactosidase was observed only in Experiment 4.1, but not in other experiments. However, both Experiment 4.1 and Experiment 4.4 showed an improvement of AME_n due to the dietary inclusion of α-galactosidase. These results are consistent with findings in other research. Swift *et al.* (1996) reported improved growth performance of broilers and improved digestibility of nitrogen and energy by the dietary supplementation of Vegpro, a mixture of protease, amylase and α-galactosidase. Zanella *et al.* (1999) showed that commercial carbohydrase products improved weight gain and feed conversion ratio in broilers fed a corn-soy diet as result of increased ileal digestibility of protein and NSPs. However, Marsman *et al.* (1997) found only apparent ileal digestibility of crude protein and NSPs, but not growth performance, of broilers was improved by treating soybean meal with protease and carbohydrase. Graham *et al.* (2002) also reported that the treatment of soybean meal with α-galactosidase reduced raffinose and stachyose by 65 and 50% respectively and increased TME from 2,974 to 3,328 kcal/kg. However, chick growth performance was not improved by enzyme treatment. Similar results were also reported by Kocher *et al.* (2002 and 2003).

Effects of Citric Acid on Growth Performance, Digesta pH, AME_n and Digestibility of DM, CP and NDF

Either no effects or negative effects of dietary citric acid on chick growth performance were observed in these studies. These results are in agreement with the previous observations (Experiments 2.2 and 2.3) and the results reported by Cave (1984), Brown and Southern (1985). However, Vogt *et al.* (1979, 1981) and Skinner *et al.* (1991) found a positive influence on chick growth performance by dietary inclusion of several of organic acids up to 2%.

Although improved digestibility of DM, CP and NDF by citric acid addition was noticed in Experiment 4.1, later studies showed that citric acid caused the depression of AME_n. Few reports of similar results were found in the poultry literature. Giesting and Easter (1985) pointed out that

dietary addition of organic acid increased gastric proteolysis and protein and amino acid digestibility in pig.

Data from Experiment 4.4 showed that the addition of 1.5% citric acid reduced the pH of the crop content by 0.8 units. This result supports the previous findings (Experiment 2.2) and the findings reported by Hinton *et al.* (2000).

Interactive Effects of α-Galactosidase and Dietary Energy Level on AME_n of the Diets

The interaction of dietary energy by α-galactosidase on AME_n of the diets is shown in Figure 6.1 (based upon the data from Table 6.20). The AME_n content increased by 11.6% when α-galactosidase was included in the low energy basal diet. However, there was no increase of AME_n when the enzyme was added in the normal energy basal diet. This result supports the earlier findings by Schang *et al.* (1997) who demonstrated that the addition of enzyme to a low nutrient density diet, but not to a high nutrient density diet, improved chick growth performance. Graham *et al.* (2002) reported that the TME of soybean meal was increased by 11.9% due to α-galactosidase treatment.

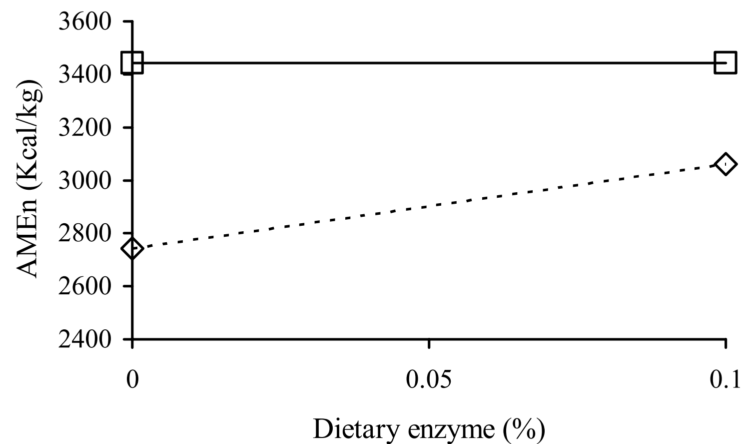

Figure 6.1. Dietary AME_n as affected by dietary α-galactosidase. □ = normal energy basal diet, ◊ = low energy basal diet. The difference in slope between two lines demonstrated the dietary energy level by enzyme interaction.

Interactive Effects of Citric Acid and Dietary Energy Level on AME_n of the Diets

Figure 6.2 shows the interaction of citric acid and dietary energy level on the AME_n of the diets (based on data from Table 6.22). The AME_n content decreased when citric acid was included in the low energy basal diets, but not in normal energy diets. This result explains the inconsistency about the effect of citric acid on chick growth performance found in previous studies. In Experiment 2.2, when a normal energy level (3010 Kcal/kg) basal diet was used, there were no negative effects on growth performance due to inclusion of citric acid up to 2.5%. However, in Experiment 4.1 and 4.2, when a low energy (2740 Kcal/kg) basal diet was used, a significant depression of chick growth performance was observed due to addition of 2% or even 1% citric acid. These results are not consistent with the results reported by Bolton and Dewar (1964) and Hume *et al.* (1993). Based on their results, many of the organic acids can serve as substrates in intermediary metabolism and can be used as energy sources.

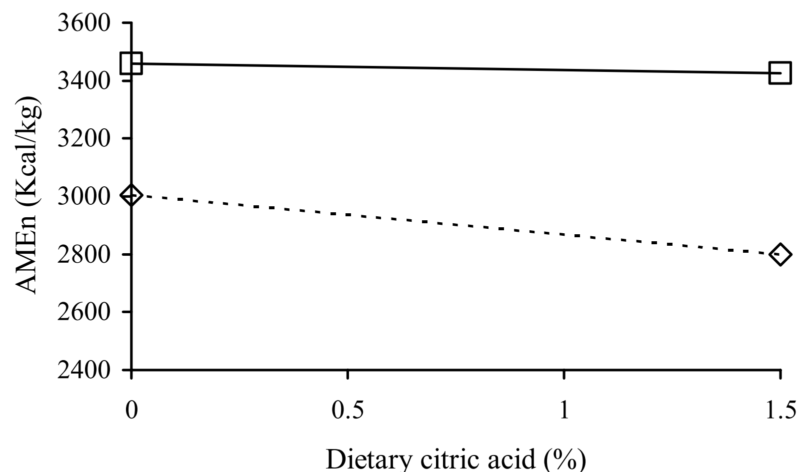

Figure 6.2. Dietary AME_n as affected by dietary citric acid. □ = normal energy basal diet, ◊ = low energy basal diet. The difference in slope between two lines demonstrated the dietary energy level by citric acid interaction.

Interactive Effects of α-Galactosidase and Citric Acid on Chick Growth Performance, Digestibility and AMEn

Interactive effects of α-galactosidase and citric acid were found in all studies. In Experiment 4.1, the depression of chick weight gain and gain to feed ratio in first two weeks due to dietary inclusion of citric acid was corrected by the supplementation of α-galactosidase. Simultaneous supplementation of α-galactosidase and citric acid significantly increased the

digestibility of DM and NDF and the AME_n of the diets, compared with the use of the control diet or the control diet with either α-galactosidase or citric alone. In Experiment 4.3 and 4.4, the chicks fed both α-galactosidase and citric acid had a higher gain to feed ratio than the chicks fed α-galactosidase or citric acid alone. Figure 6.3 clearly shows the interactive effects of α-galactosidase and citric acid on AME_n of the diets. These data are from Experiment 4.4 and listed in Table 6.21. The AME_n content decreased when citric acid is included in the diet in the absence, but not in the presence of α-galactosidase. Previous studies showed that α-galactosidase increased the release of reducing sugars from SBM *in vitro* (Experiment 3.1 and 3.2) and improved AME_n of the corn-soy diet (Experiment 4.1).

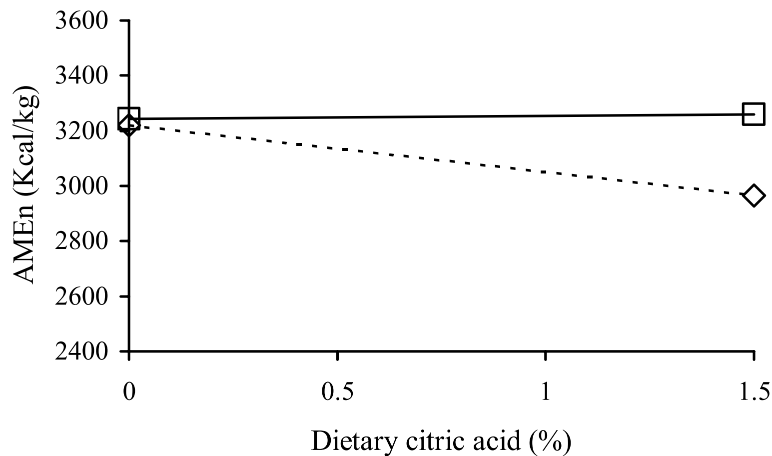

Figure 6.3. Dietary AME_n as affected by dietary α-galactosidase and citric acid. □ = + 0.1% α-galactosidase, ◊ = no α-galactosidase. The difference in slope between two lines demonstrated the dietary α-galactosidase by citric acid interaction.

CHAPTER 7: SUMMARY AND CONCLUSIONS

The objective of this research was to investigate the effects of acidification of broiler diets on the efficacy of exogenous enzymes. Four series of studies were conducted. Food grade citric acid was used as an acidifier. Five commonly used feed-grade enzyme products (β-glucanase, xylanase, amylase, protease and α-galactosidase) were evaluated in the first study. Based on the results from first study, later studies focused mainly on the effects of α-galactosidase, protease and amylase on *in vitro* and *in vivo* digestion, inactivation of anti-nutritional factors in soybean and growth performance of broiler chicks fed corn-soybean meal-based diets.

Effects of pH Levels Commonly Found in Avian Digestive Tract on *In Vitro* Activity of Feed-Grade Enzyme Products

The purpose of Study 1 was to measure the activities of feed-grade enzyme products at pH levels simulating various parts of the avian digestive tract. Beta-glucanase, xylanase, amylase, α-galactosidase and protease were assayed in triplicate at their optimum pH levels and at pH levels of 3.0, 6.0, 6.5, 7.0 and 7.5, which were used to simulate pH levels found in the gizzard, the diet, the crop, and the proximal and distal parts of small intestine, respectively. The pH gradient was obtained by extracting the enzymes in different buffers. Results showed that the optimal pH for four of five enzymes (β-glucanase, xylanase, amylase and α-galactosidase, but not protease) was around 5.0 and all the enzymes had reduced activity at pH 6.5 or above. Very low activity or no activity was evident for xylanase, α-galactosidase and amylase at pH 3.0. Protease had highest activity at pH 3.0, but it had very low or no activity at pH 6.0 or above. The data suggest that the pH levels commonly found in different segments of the avian digestive tract may be a limiting factor for maximum activity of the exogenous enzymes, such as amylase, α-galactosidase and protease.

Effects of Dietary Organic Acids on the pH of the Diet and Digesta and the Growth Performance of Broiler Chicks

The purpose of Study 2 was to investigate the effects of dietary supplementation of organic acids on the pH of the diet and digesta and the growth performance of broiler chicks. This study included three experiments (Experiments 2.1, 2.2 and 2.3).

In Experiment 2.1, five different organic acids were added to a corn-soybean meal-based diet at six different levels. Dietary inclusion of graded levels of organic acids linearly reduced ($P<0.001$) the pH of the diet. Different organic acids had different ($P<0.001$) effects, with formic acid and citric acid being most effective in lowering dietary pH.

Experiment 2.2 and 2.3 were animal studies. In Experiment 2.2, chicks were fed either a corn basal diet with five different levels of citric acid (0 to 2.5%) or a barley or a wheat basal diet with or without 1.5% citric acid. Graded levels of citric acid in the diet resulted in a linear reduction ($P<0.01$) of the pH of the crop content, but did not affect the pH of digesta in the gizzard or in the small intestine. Chick growth performance was not improved by either including graded levels of citric acid (0 to 2.5%) in corn-soy basal diet or including 1.5% citric acid in the basal diets containing different grain sources.

In Experiment 2.3, chicks were fed either a low energy or a normal energy corn-soybean meal-basal diet with or without 2% citric acid or fumaric acid. Dietary inclusion of citric acid or fumaric acid caused a negative effect ($P<0.01$) on chick growth performance.

In Vitro Evaluation of Simultaneous Application of Citric Acid and Exogenous Enzymes on Nutrient Release from Soybean Meal and Trypsin Inhibitor Content in Raw Whole Soybean

Study 3 used an *in vitro* approach to evaluate the effects of simultaneous application of citric acid and α-galactosidase or protease on *in vitro* nutrient release from soybean meal and trypsin inhibitor content in raw whole soybeans. An *in vitro* model was used to simulate the chicken's digestive process in the crop, the gizzard and the small intestine. Soybean meal and raw whole soybean were used as substrates. Graded levels of either α-galactosidase (0 to 13,792 units/kg) or protease (0 to 888 units/kg) and 0 or 2% citric acid were added to the substrates in a factorial arrangement of treatments. Reducing sugars and α-amino nitrogen were measured at the

end of the crop phase, the gizzard phase, and the whole phase (crop through small intestine). Trypsin inhibitor content was measured at the end of the gizzard phase. The study indicated that increasing levels of α-galactosidase linearly increased ($P<0.001$) the release of the reducing sugars from soybean meal in both the crop and whole phases. Addition of citric acid further increased ($P<0.001$) the activity of α-galactosidase, resulting in a significant interaction. Increasing the levels of protease linearly increased ($P<0.001$) the α-amino nitrogen release from soybean meal and raw whole soybean in all phases. However, trypsin inhibitor content in the raw whole soybean was not influenced by the application of the protease.

Effects of Dietary Supplementation of Enzymes and Acidification of Diet and Water on Nutrient Digestibility and Chick Growth Performance

In Study 4, four experiments (Experiments 4.1, 4.2, 4.3 and 4.4) were conducted to examine the effects of dietary supplementation of α-galactosidase, amylase and acidification of the diet and drinking water on nutrient digestion and growth performance of broiler chicks. Low energy (2,740 Kcal/kg) or normal energy (3,110 Kcal/kg) corn-soybean meal basal diets were used in these experiments.

In Experiment 4.1, dietary treatments consisted of feeding a low energy basal diet alone or supplemented with 1724 units/kg α-galactosidase or 2% citric acid or both. Dietary α-galactosidase increased feed intake ($P<0.01$), weight gain ($P<0.05$), AME_n of the diets ($P<0.05$) and digestibility of CP ($P<0.05$) and NDF ($P<0.01$). Citric acid increased the digestibility of DM ($P<0.01$), CP ($P<0.05$) and NDF ($P<0.01$), but decreased feed intake ($P<0.01$) and weight gain ($P<0.01$). Simultaneous supplementation of α-galactosidase and citric acid further increased AME_n of the diets ($P<0.05$) and digestibility of DM ($P<0.05$) and NDF ($P<0.01$).

In Experiment 4.2, the low energy basal diet was fed alone or with a mixture of 1724 units/kg α-galactosidase and 9705 units/kg amylase, with 1% citric acid, or with both enzymes plus acid. No improvements of growth performance and nutrient digestibility were found due to dietary inclusion of enzyme, citric acid or both.

In Experiment 4.3, chicks were given two dietary levels of citric acid (0 or 0.5%), two dietary levels of α-galactosidase (0 or 6896 units/kg) and two water sources (normal or acidified water) in a 2 x 2 x 2 factorial arrangement of treatments. Water was acidified by a commercial organic acidifier. Water acidification had no influence on chicks' water consumption. No effects

of dietary α-galactosidase and water acidification on growth performance and nutrient digestibility were observed. Dietary citric acid depressed AME_n of the diet ($P<0.01$). A significant interaction ($P<0.01$) of α-galactosidase and citric acid on growth performance was found. The birds fed citric acid or α-galactosidase alone had lower ($P<0.01$) body weight gain and gain to feed ratio ($P<0.01$) than the birds fed the basal diet alone or the basal plus both citric acid and α-galactosidase. The chicks fed both citric acid and acidified water had lower digestibility of DM ($P<0.05$) and CP ($P<0.01$) than those fed citric acid or acidified water alone resulting in a significant interaction.

In Experiment 4.4, chicks were fed diets with two levels of energy (low or normal), two levels of citric acid (0 or 1.5%) and two levels of α-galactosidase (0 or 1724 units/kg) in a 2 x 2 x 2 factorial arrangement of treatments. Dietary α-galactosidase increased ($P<0.01$) reducing sugar concentration in the crop content and improved ($P<0.01$) the AME_n of the diets. Dietary citric acid decreased ($P<0.01$) the pH of the crop content and increased ($P<0.05$) the activity of the α-galactosidase. Citric acid decreased ($P<0.01$) the AME_n of the diet. The reduction of AME_n due to the supplementation of citric acid occurred only when citric acid was added to the diets without α-galactosidase, but not to the diets with α-galactosidase. Chicks fed citric acid had lower ($P<0.05$) gain to feed ratio than did the chicks fed both α-galactosidase and citric acid. The inclusion of α-galactosidase in the low energy basal diets caused a 11.6% improvement ($P<0.01$) of AME_n, while the addition of α-galactosidase to the normal energy basal diet had no effect on the AME_n of the diets. The inclusion of citric acid in the low energy basal diets caused a 6.9% reduction ($P<0.01$) of AME_n, while the addition of citric acid to the normal energy basal diet had no effect on the AME_n of the diets.

Implications

Supplementation of α-galactosidase, which digests anti-nutritional galactosides in soybean meal, can benefit broiler chicks by improving AME_n of the diets and, in turn, growth performance. The beneficial effect of α-galactosidase is closely related to energy levels of diets. Based on the results obtained from this study, energy can be lowered up to 10% when α-galactosidase is included in the diet.

Organic acids are widely used to inhibit pathogenic bacteria (*e.g., Salmonella spp.*) in both raw materials and finished poultry feed. However, negative effects on growth performance by

dietary inclusion of organic acids were observed in this study and in many others reported in the literature. Very interestingly, these negative effects caused by organic acid supplementation were corrected by simultaneously using α-galactosidase. Results from this study also showed that the negative effects of organic acid are related to the energy content in the diet. The negative effect of organic acid on AME_n of the diets can be alleviated either through increasing the energy level of the diet or through using α-galactosidase simultaneously. On the other hand, both *in vitro* and *in vivo* data in this study indicated an enhancement of α-galactosidase activity by simultaneously using organic acid. These results suggest that simultaneously using α-galactosidase and organic acid is more beneficial to nutrient digestibility and growth performance of broiler chicks than using α-galactosidase or organic acid alone.

Potential Economic Benefits

A model was created to estimate potential economic benefits of using α-galactosidase in broiler diets. The model is based on the following assumptions and conditions:

1) a low energy diet containing 2.80 Mcal/kg was formulated using a least cost feed formulation program (Diet 1, Table 7.1);
2) the diet contained 1.5% citric acid to help control pathogenic microorganisms;
3) the addition of α-galactosidase may improve the AME_n of the diet by 5, 10 or 15%;
4) for simplification, it is assumed that savings in grower and finisher periods would be similar to those observed in the starter phase;
5) to obtain improvement of 5, 10 and 15% in the AME_n of the diet without using α-galactosidase, Diets 2, 3, and 4, respectively, were formulated using commonly used ingredients (Table 7.1).
6) ingredient prices were based on current market costs.

Table 7.2 illustrates the potential savings from using α-galactosidase in a low energy diet using this model. If the improvement in AME_n due to enzyme supplementation is 5%, no savings are realized because it is possible to formulate a diet with 5% more AME_n (Diet 2) that does not cost more than Diet 1 plus the cost of the enzyme ($8/MT). However, if increases in AME_n of 10 or 15% are obtained with α-galactosidase, then the respective savings would be $7.90 and $15.40 per MT. The results from the current study (Experiment 4.4) showed an 11.6% increase of AME_n by using α-galactosidase in a low energy diet (Table 6.20). Therefore, savings by using α-

galactosidase of \$7.90/MT due to a 10% increase in AME_n might be reasonable. A flock of 25,000 broilers would consume a total of approximately 102 MT of feed during 42 d period. Thus, total savings in feed costs due to a 10% increase in AME_n from enzyme supplementation would be \$ 806/flock. If this figure is extrapolated to a broiler complex producing 50,000,000 birds per year, the annual savings would be \$1,612,000.

Limitations

There are several limitations of this research work. In order to measure nutrient digestibility, all animals in present studies were housed in wire-floored cages in a university research farm and studies were ended by either 14 or 21 d. However, in commercial production, broilers are grown for longer period (*e.g.*, 42 d). It is not known if the results from a 14 d or 21 d growth trail can be extrapolated to the full growth period. Also, commercial broilers are typically grown on the floor with some type of bedding (*e.g.*, rice hulls or wood shavings) in much larger facilities. Under commercial condition, birds can practice coprophagy and are likely exposed to more environmental stresses, especially microbial stress. Birds in the present studies were not given a microbial challenge that is essential to test the anti-microbial effect of organic acids. Based on the above limitations, the further studies should be conducted to investigate the effect of simultaneous application of organic acid and α-galactosidase on the growth performance of broiler chicks to market age under conditions that are similar to those used in commercial practice.

Table 7.1. Formulas and costs of four corn-soy diets with different AME_n[1]

Ingredient	Price ($/100kg)	Diet 1 AME_n (2.80 Mcal/kg)	Diet 2 AME_n (2.95 Mcal/kg)	Diet 3 AME_n (3.09 Mcal/kg)	Diet 4 AME_n (3.22 Mcal/kg)
		Formula (%)	Formula (%)	Formula (%)	Formula (%)
Corn	7.59	52.60	52.60	51.35	50.12
Soybean meal (48% CP)	16.10	36.00	37.00	38.00	38.70
Wheat middlings	5.62	7.50	4.19	1.90	-
Blended animal-vegetable fat	34.18	-	2.30	4.80	7.20
Salt	6.62	0.43	0.44	0.45	0.45
Limestone	8.82	1.38	1.35	1.33	1.33
Dicalcium phosphate	30.87	1.66	1.70	1.74	1.76
Vitamin-mineral mix	264.60	0.25	0.25	0.25	0.25
DL-Methionine	330.75	0.18	0.18	0.19	0.19
Total		100	100	100	100
Cost ($/MT[2])		121.30	128.80	137.20	144.70

[1]All diets were formulated to provide a minimum of 22.70% CP, 0.45% available P, 1.00% Ca, 0.53% methionine, 0.90% methionine + cystine, 1.27% lysine and 0.20% sodium.

[2]metric ton.

Table 7.2. Potential savings from using α-galactosidase in a low energy broiler diet

Diet	AME_n (Mcal/kg)	Increase (%)	Cost ($/MT)	Increase ($/MT)	Savings by using enzyme ($/MT)[1]
1	2.80	0	121.30	0	
2	2.95	5	128.80	7.50	-0.50
3	3.09	10	137.20	15.90	7.90
4	3.22	15	144.70	23.40	15.40

[1]Increased cost – enzyme cost ($8/mt feed)

APPENDIX 1: DETERMINATION OF β-GLUCANASE ACTIVITY

Reference:

Miller, G. L., 1959. Anal. Chem. 31:426-428.

Bathgate, G. N., 1979. Journal of the Institute of the Brewing. 85:92-94.

Principle:

Beta-glucanase is allowed to hydrolyze β-glucan under the assay conditions. Reducing sugars are formed during the hydrolysis. One activity unit (BGU) is defined as that quantity of enzyme that will liberate 1 μmol of reducing sugars (expressed as glucose) in one minute under the assay conditions.

Apparatus:

Spectrophotometer, pH meter, magnetic stirrer with hotplate, water bath, stopwatch, volumetric flasks, beakers, test tubes, pipettes and glass fiber filter paper.

Reagents and Solutions:

a) 1.0% (W/W) β-glucan substrate: weigh 1.0 g β-glucan in a beaker and mix with 10 ml ethanol. Add 80 ml of cold deionized water. Stir the mixture on a magnetic stirrer and increase the temperature to boiling. Stir the mixture vigorously until β-glucan is completely dissolved. Cool the solution to room temperature by continuously stirring. When cooled, adjust the concentration to 1.0 % (w/w) by addition of deionized water. Filter through glass fiber filter paper. Substrate can only be used on the day it is prepared.

b) 0.1 M sodium acetate buffer, pH 5.0: dissolve 8.2 g of anhydrous sodium acetate in deionized water; adjust to 1000 ml (0.1 M). Dissolve 6.0 g of glacial acetic acid in deionized water; adjust to 1000 ml (0.1 M). Adjust the pH of sodium acetate solution with 0.1 M acetic acid to 5.0.

c) Sample solution: dissolve 0.1-0.5 g of enzyme powders to 80 ml of 0.1 M Na-acetate buffer, pH 5.0. Stir 15 min. Bring the volume to 100 ml. Let the solution stand for at least 1 h at room temperature before further dilutions or measurements are made. The final activity of the solution should be about 0.12-0.22 U/ml.

d) Dinitrosalicylic acid (DNS) reagent: suspend 20.0 g of dinitrosalicylic acid in 400 ml water and gradually add 300 ml of NaOH solution containing 32.0 g NaOH with continuous stirring. Warm the suspension in water bath at 50ºC until it is clear. Do not exceed 50ºC. Gradually add 600 g potassium sodium tartrate ($KNaC_4H_4O_6{\cdot}4H_2O$) to the solution with continuously stirring. Dilute the solution to 2000 ml with deionized water and filter through a glass filter. Store the solution in a dark bottle at room temperature.

Procedure:

1. Standard Curve:
   a) Glucose stock solution: dissolve 1.000 g of glucose (dry-weight basis; *e.g.*, 2 h at 105ºC) in 100 ml of deionized water. Mix thoroughly.
   b) Glucose working standard solutions: pipette 2 ml, 4ml, 6ml, 8 ml and 10 ml of stock solution to volumetric flasks and bring to 100 ml using deionized water. Mix thoroughly.
   c) Pipette 1 ml of each work solution, 1 ml of deionized water and 3 ml of DNS reagent in test tubes (= standard samples). Pipette 2 ml of deionized water instead of the above mentioned standard and deionized water and 3 ml of DNS-reagent in test tubes (= reagent blank). Stir all the tubes carefully and boil together for exactly 5 min. The water must boil all the time. Stop the color development by cooling the reaction mixtures in an ice-cold water bath and allow to equilibrate to room temperature. Stir the tubes and measure absorbance at 540 nm against water. Plot the standard curve using absorbance vs. concentration. . In order to minimize variation, it is advisable to construct a standard curve for every series of assays. Also, samples and standard curves should be prepared, boiled, and read at the same time.
2. Sample Measurement:
   a) Equilibrate 1.0 ml of β-glucan substrate at 30˚C for 10 min. Allow two tubes for each sample, two for each enzyme blank and two for the substrate blank.
   b) At time zero, rapidly pipette 1.0 ml of an appropriate enzyme dilution solution into the equilibrated substrate tube, but not the enzyme blank and substrate blank tubes. Mix thoroughly.
   c) Incubate for 10 min at 30˚C water bath.

d) After exactly 10 min, add 3.0 ml of DNS solution to each tube, mix and remove the tubes from the water bath.
e) Add 1.0 ml of enzyme dilution to the enzyme blank and 1.0 ml of buffer to the substrate blank.
f) Boil the reaction mixture for exactly 5 min. Stop the color development by cooling the reaction mixtures in ice-cold water bath for 5 min. Equilibrate the tubes to room temperature.
g) Measure the absorbance of each sample and blank against the deionized water at 540 nm.

Calculation:

The β-glucanase activity is obtained by using the formula:

$$\text{Activity} = \frac{(\Delta A - \text{intercept})}{\text{Slope}} \times \frac{(1000 \times Df)}{MW_{glucose} \times t}, \ \mu\text{mol/ml per minute}$$

$\Delta A = A_{\text{enzyme sample}} - A_{\text{enzyme blank}}$

Slope = the slope of the standard curve

Intercept = the intercept of the standard curve

MW = molecular weight of anhydrous glucose (180.16 g)

t = enzyme reaction time, 10 min.

1000 = factor milligrams to micrograms

Df = dilution factor

APPENDIX 2: DETERMINATION OF XYLANASE ACTIVITY

Reference:

Bailey M. J., and K. Poutanen, 1989. Appl. Microbiol. Biotechnol. 30:5-10.

Principle:

This assay is based on a 5 minute hydrolysis of xylan substrate at pH 5.3 and 50°C. One xylanase unit is the amount of enzyme which liberates 1 μmol of xylose per minute under the conditions of the assay.

Apparatus:

Spectrophotometer, pH meter, magnetic stirrer with hotplate, water bath, stopwatch, volumetric flasks, beakers, test tubes, pipettes and glass fiber filter paper.

Reagents and Solutions:

a) 0.05 M sodium citrate buffer, pH 5.3: dissolve 6.45 g of sodium citrate ($C_6H_5Na_3O_7$, MW 258) in 500 ml of deionized water (0.05M). Dissolve 2.4 g of citric acid ($C_6H_8O_7$, MW 192) in 250 ml of deionized water (0.05M). Adjust the pH of sodium citrate solution with 0.05M citric acid to 5.3.

b) Xylan substrate: heat 80 ml of buffer to boiling point on a heating magnetic stirrer. Remove from heat to another stirrer and add 1.000 g of xylan (Sigma # X -0502). Cool with continued stirring, cover and stir slowly overnight. Make up to 100 ml with buffer. This solution is stable for 7 days when refrigerated.

c) Xylose stock solution, 0.01 M: dissolve 0.15 g of D-xylose in 100 ml of buffer. Mix thoroughly. Xylose stock solution must be made fresh daily.

d) Dinitrosalicylic acid (DNS) reagent: suspend 10 g DNS in 400 ml of deionized water and gradually add 150 ml of sodium hydroxide solution (16 g NaOH per 150 ml of water) while mixing. Warm the suspension in a water bath at 50°C until it is clear. Do not exceed 50°C. Gradually add 300 g potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) to the solution with continuous mixing. Dilute the solution to 1 L with deionized water and filter through

a glass filter. Store the solution in a dark bottle at room temperature. The solution is stable for 6 months.

Procedure:

1. Equilibrate 1.8 ml of xylan substrate at 50°C for 5 min. Allow two tubes for each sample, two for each enzyme blank and two for the substrate blank.
2. At time zero, rapidly pipette 0.2 ml of an appropriate enzyme dilution solution into the equilibrated substrate except enzyme and substrate blank tubes. Mix thoroughly.
3. Incubate for 5 min at 50°C.
4. After exactly 5 min, add 3 ml of DNS solution to each tube, mix and remove the tubes from the water bath.
5. Add 0.2 ml of enzyme dilution to the enzyme blank and 0.2 ml of buffer to the substrate blank.
6. Boil the reaction mixture for 5 min. Stop the color development by cooling the reaction mixtures in ice-cold water bath for 5 min. Equilibrate the tubes to room temperature.
7. Measure the absorbance of each sample and blank against the deionized water at 540 nm.

Standard Curve:

Dilute the stock solution in buffer as follows:

Undiluted = 10.0 μmol/ml
1:2 = 5.0 μmol/ml
1:4 = 2.5 μmol/ml
1:5 = 2.0 μmol/ml

To set up the standard curve, treat the standard solution exactly the same way as the substrate blank. Put 1.8 ml substrate plus 0.2 ml each standard to the test tube. Add 3 ml of DNS solution to each tube, mix and incubate for 5 min. Then remove the tubes from the water bath and boil for 5 min and cool for 5 min in a cold water bath. Read the absorbance against the deionized water at 540 nm. Plot the standard curve using absorbance vs. concentration. In order to minimize variation due to different batches of DNS, boiling conditions, substrate solution, automatic pipette calibration and even the day and hour of assay, it is advisable to construct a

standard curve for every series of assays. Also, samples and standards should be prepared, boiled, and read at the same time.

Calculation:

Take the corresponding concentration (= ΔC) to corrected absorbance (ΔA = absorbance of sample minus absorbance of corresponding blank) from the standard curve. The enzyme activity will be calculated based on the following formula:

$$\text{Activity} = \frac{\Delta C \text{ x dilution factor}}{5}\text{; } \mu\text{mol/ml per minute}$$

APPENDIX 3: DETERMINATION OF α-AMYLASE ACTIVITY

Reference:

FCC: Food Chemicals Codex , WA 701 N277f, 1996.

Principle:

Alpha-amylases breakdown the alpha 1-4 glucosidic linkages of dextrin to yield maltose and smaller dextrins. The broken-down products react with an iodine solution and the color produced is compared to a standard color solution. As starch is broken down the color changes from blue to red-brown. The enzyme activity is expressed as FAU units.

One FAU unit is the amount of enzyme which will dextrinize soluble starch at the rate of 1 mg per min at 30°C and pH 4.8.

Apparatus:

Spectrophotometer, pH meter, magnetic stirrer with hotplate, water bath, stopwatch, volumetric flasks, beakers, test tubes and pipettes.

Reagents and Solutions:

a) 2 M acetate buffer: dissolve 164 g of anhydrous sodium acetate in about 500 ml of distilled water. Add 120 ml of glacial acetic acid and adjust pH to 4.8 with glacial acetic acid. Dilute to 1 L with distilled water and mix thoroughly.
b) Buffered starch solution: disperse 2.0 g of potato soluble starch (J. T. Baker or equivalent) in a 20 ml of distilled water and pour slowly into 60 ml of boiling water. Boil with stirring for 1 - 2 min. Quantitatively transfer the mixture to a 100 ml volumetric flask with the aid of water. Add 5 ml of acetate buffer (pH 4.8) and dilute to volume with water. Prepare fresh daily.
c) Enzyme dilution solution: in a 1 L volumetric flask, dissolve 0.585 g sodium chloride and 2.22 g calcium chloride to 800 ml distilled water. Add 20 ml of 2 M acetate buffer; adjust pH to 4.8 with 1 M NaOH and fill to the volume.

d) Stock iodine solution: dissolve 1.1 g iodine and 2.2 g potassium iodide in 25 ml distilled water, transfer to a 50 ml volumetric flask and fill to the volume. Store the solution in darkness. Prepare fresh solution monthly.

e) Working iodine solution: dissolve 10 g of potassium iodide in 200 ml distilled water; add 1.0 ml of stock iodine solution and fill to 250 ml with distilled water. Prepare fresh daily.

f) Standard color solution: dissolve 25.0 g cobaltous chloride ($CoCl_2 \cdot 6H_2O$) and 3.84 g potassium dichromate in 100 ml 0.01 N hydrochloric acid.

Procedure:

1. Dilute enzyme samples in enzyme dilution solution as to give an end point between 10 to 20 min in the procedure below.
2. For each enzyme sample to be analyzed, place 5 ml of buffered starch solution into a 20 mm x 150 mm test tube and allow to equilibrate in a 30ºC water bath for 5 to 10 min.
3. For each enzyme sample to be analyzed, dispense 5 ml of working iodine solution into 5 - 15 separate tubes. Place the tubes in the 30°C water bath.
4. Warm the final enzyme dilution in a 30ºC water bath to equilibrium.
5. Zero the spectrophotometer (617 nm) using distilled water and record the absorbance of the standard color solution. Absorbance should be about 0.410.
6. Transfer 2.5 ml of the enzyme solution into starch flask and mix. Begin timing the reaction and continue to incubate at 30°C.
7. After 9 to 10 minutes of reaction and at definite time intervals (about 30 sec), place 1 ml of reaction mixture into one of the 5 ml working iodine solutions described above, mix and record optical density (O.D.).
8. As the O.D. of the reaction mixture approaches that of the color standard, measure the O.D. of the reaction mixture every 30 seconds until the O.D. matches the color standard or until a regression curve can be made that includes the O.D. of the color standard. This time is the end point of the reaction.

Calculations:

$$\frac{40 \times F}{T}$$

Where: 40 = a constant derived from the 100 mg of starch (5 ml of a 2% solution) and the 2.5 ml aliquot of sample preparation used (100/2.5).

T = time of reaction in minutes.

F = dilution factor.

APPENDIX 4: DETERMINATION OF PROTEASE ACTIVITY

Reference:

FCC: Food Chemicals Codex , WA 701 N277f, 1996

Principle:

The assay is based on a 30 min enzymatic hydrolysis of a Hammarsten casein substrate at pH 3.0 and 37°C. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The quantity of solubilized casein in the filtrate is determined spectrophotometrically. The proteolytic activity is expressed as spectrophotometric acid protease units (SAP) with 1 unit equal to the amount of enzyme that liberates 1 μmol of tyrosine per min from casein under the conditions specified.

Apparatus:

UV spectrophotometer, pH meter, magnetic stirrer with hotplate, water bath, stopwatch, volumetric flasks, beakers, test tubes, pipettes and Whatman No. 42 filter paper.

Reagents and Solutions:

a) Glycine-hydrochloric acid buffer (0.05 M): dissolve 3.75 g of glycine in about 800 ml of distilled water. Adjust pH of the solution to 3.0 with 1 N hydrochloric acid. Quantitatively transfer the solution to a 1000 ml volumetric flask, dilute to the volume with distilled water, and mix thoroughly.
b) Trichloroacetic acid (TCA) solution: dissolve 18.0 g trichloroacetic acid and 11.45 g anhydrous sodium acetate into 800 ml distilled water, and add 21.0 ml of glacial acetic acid. Quantitatively transfer the solution to a 1000 ml volumetric flask, dilute to volume with water, and mix thoroughly.
c) Substrate solution: pipette 8 ml 1 N hydrochloric acid into 500 ml distilled water, and disperse 7.0 g (moisture-free basis) Hammarsten-grade casein (United States Biochemical Corp., Catalog No. 12840, or equivalent) into this solution with continuous agitation. Heat for 30 min in a boiling water bath, stirring occasionally, and cool to room temperature. Dissolve 3.75 g of glycine in the solution, and adjust the pH of the solution

to 3.0 with 0.1 N hydrochloric acid. Quantitatively transfer the solution to a 1000 ml volumetric flask, dilute to volume with distilled water, and mix thoroughly.

d) Sample preparation: prepare the sample enzyme solution using glycine-hydrochloric acid buffer so that 2 ml of the final dilution will give a corrected absorbance of enzyme incubation filtrate at 275 nm (ΔA, as defined in the Procedure) between 0.200 and 0.500. Weigh the enzyme preparation, quantitatively transfer it to a glass mortar, and triturate with glycine-hydrochloric acid buffer. Quantitatively transfer the mixture to an appropriately sized volumetric flask, dilute to volume with glycine-hydrochloric acid buffer, and mix thoroughly.

Procedure:

1. Pipette 10.0 ml of substrate solution into each of a series of 25 x 150 mm test tubes, allowing at least two tubes for each sample, one for each enzyme blank, and one for a substrate blank.
2. Stopper the tubes, and equilibrate them for 15 min in a water bath maintained at 37°C.
3. At zero time, start the stopwatch, and rapidly pipette 2.0 ml sample enzyme preparation into the equilibrated substrate. Mix by swirling, and replace the tubes in the water bath. Add 2 ml glycine-hydrochloric acid buffer instead of the sample preparation to the substrate blank.
4. After exactly 30 min, add 10 ml of TCA solution to each of enzyme incubations and to the substrate blank to stop the reaction. In the following order, prepare an enzyme blank containing 10 ml of substrate solution, 10 ml of TCA solution, and 2 ml of the sample preparation. Heat all tubes in the water bath for 30 min, allowing the precipitated protein to coagulate completely.
5. At the end of the second heating period, cool the tubes in an ice bath for 5 min, and filter through Whatman No. 42 filter paper, or equivalent. The filtrate must be perfectly clear.
6. Determine the absorbance of each filtrate in a 1 cm cell at 275nm with a suitable spectrophotometer, against the substrate blank. Correct each absorbance by subtracting the absorbance of the respective enzyme blank.

Standard Curve: Transfer 181.2 mg of L-tyrosine, chromatographic-grade or equivalent (Sigma Chemical Co.), previously dried to constant weight, to a 1 L volumetric flask. Dissolve in 60 ml of 0.1N hydrochloric acid. When the L-tyrosine is completely dissolved, dilute the solution to the volume with distilled water, and mix thoroughly. This solution contains 1.00 μmol of tyrosine per 1.0 ml. Prepare dilutions from this stock solution to contain 0.10, 0.20, 0.30, 0.40, and 0.50 μmol/ml. Measure against a water blank the absorbance of each dilution in a 1 cm cell at 275 nm. Prepare a plot of absorbance versus μmol of tyrosine per ml. A straight line must be obtained. Determine the slope and intercept for use in the calculation below.

Calculation:

$$SAP/g = (\Delta A - I) \times 22 / (S \times 30 \times W),$$

in which ΔA is the corrected absorbance of the enzyme incubation filtrate; I is the intercept of the standard curve; 22 is the final volume of the incubation mixture, in ml; S is the slope of the standard curve; 30 is the incubation time, in min; and W is the weight, in g, of the enzyme sample contained in the 2.0 ml aliquot of sample preparation added to the incubation mixture in the procedure.

APPENDIX 5: DETERMINATION OF α-GALACTOSIDASE ACTIVITY

Reference:

Ratto, M., and K. Poutanen, 1988. Biotachnol. Lett. 10:661-664.

Ademark, P., M. Larsson, F. Tjerneld, and H. Stalbrand, 2001. Enzyme and Microbial Technology 29:441-448.

Principle:

The assay is based on a 5 min hydrolysis of ρ-nitrophenyl-α-D-galactopyranoside followed by spectrophotometric measurement of the liberated ρ-nitrophenol at 405 nm. One galactosidase activity unit (GalU) is defined as the quantity of the enzyme that will liberate ρ-nitrophenol at the rate of 1 μmol/min under the conditions of assay.

Apparatus:

Spectrophotometer, pH meter, magnetic stirrer with hotplate, water bath, stopwatch, volumetric flasks, beakers, test tubes, pipettes.

Reagents and Solutions:

a) 0.1 M sodium acetate buffer solution, pH 5.0: dissolve 8.2 g of anhydrous sodium acetate in distilled water; adjust to 1000 ml (0.1 M). Dissolve 6.0 g of glacial acetic acid in distilled water; adjust to 1000 ml (0.1 M). Adjust the pH of sodium acetate solution to 5.0 with 0.1 M acetic acid.

b) Sample solution: weigh 1.000 g enzyme into 100 ml volumetric flask, and diluted to 100 ml using acetate buffer solutions. Stir 15 min. Set 1 hr. Take 1 ml supernatant, and dilute to 100 ml.

c) Substrate solution: dissolve 0.0383 g of ρ-nitrophenyl-α-D-galactopyranoside (Sigma Chemical Co., Catalog No. 877) in sodium acetate buffer solution (pH 5.0), and dilute to 100 ml.

d) 1 M Na_2CO_3 solution: dissolve 10.6 g Na_2CO_3 into distilled water, and dilute to 100 ml.

e) ρ-Nitrophenol stock solution: dissolve 0.0139 g of ρ-nitrophenol (Sigma Chemical Co., Catalog No. 104-8) in distilled water, and dilute to 100 ml. This solution contains 1 μmol/ml.

Procedure:

1. Equilibrate the enzyme solution in a water bath at 50°C for at least 5 min.
2. Transfer 1.8 ml of substrate solution into test tubes. Allow two tubes for each sample, two for enzyme blank and two for substrate blank. Equilibrate in water bath at 50°C for 5 min.
3. At zero time, add 0.2 ml of enzyme solution to the sample test tubes; add 0.2 ml acetate buffer solution to the substrate blank test tubes and add nothing to enzyme blank tubes. Mix all tubes thoroughly, and return to the water bath.
4. After exactly 5 min, add 1 ml of 1M Na_2CO_3 solution to each tube, mix, and remove from the water bath.
5. Add 0.2 ml of sample enzyme solution to each of enzyme blank test tubes, and mix thoroughly.
6. Measure the absorbance of each sample and blank at 405 nm versus that of distilled water.

Standard curve:

Prepare the following dilutions of ρ-nitrophenol stock solution with distilled water: 10 ml plus 4 ml of water (0.714 μmol/ ml), 10 ml plus 15 ml of water (0.4 μmol/ ml) and 10 ml plus 50 ml of water (0.167 μmol/ ml). Transfer 1.8 ml of the substrate solution to each of ten separate test tubes (13 x 100 mm). Add 0.2 ml of the ρ-nitrophenol stock solution to the first two tubes, 0.2 ml of each diluted solution to the next six tubes with two tubes for each solution, and 0.2 ml of distilled water to the last two tubes (substrate blank). Add 1 ml of 1 M Na_2CO_3 solution to each tube, and mix thoroughly. Read the absorbance against the deionized water at 405 nm. Plot the standard curve using absorbance vs. concentration. In order to minimize variation, it is advisable to construct a standard curve for every series of assays. Also, samples and standard curve should be prepared, incubated, and read at the same time.

Calculations:

Take the corresponding concentration (=ΔC) to corrected absorbance (ΔA = absorbance of sample minus absorbance of corresponding blank) from the standard curve.

Activity = (ΔC x dilution factor) / 5; μmol/g per minute.

APPENDIX 6: DETERMINATION OF α-AMINO NITROGEN

Reference:

Moore, S., and W. Stein, 1954. J. Biol. Chem. 211(2):907-13.

Apparatus:

Spectrophotometer, water bath, stopwatch, volumetric flasks, beakers, test tubes, pipettes.

Reagents and Solutions:

a) Ninhydrin color regent: dissolve 3.97 g anhydrous sodium phosphate (Na_2HPO_4), 6.0 g potassium phosphate (KH_2PO_4), 0.5 g ninhydrin, and 0.3 g fructose in distilled water, and bring to a volume of 100 ml.
b) Dilution solution: dissolve 2.0 g potassium iodate (KIO_3) in 600 ml of distilled water. After complete dissolution, add 400 ml 200 proof ethanol (96% ethanol is sufficient for mash samples).
c) Glycine stock solution: dissolve 107.2 mg glycine in distilled water and bring to a volume of 100 ml. Store this in a refrigerator.
d) Glycine standard solution: dilute 1 ml of glycine stock solution to 100 ml with distilled water. This standard contains 2 mg of α-amino nitrogen per liter. Make fresh daily.
e) Sample solution: dilute sample solution with distilled water to the range surrounding the glycine standard solution (generally 1:500).

Procedure:

1. Transfer 2 ml glycine standard solution to first row of screw cap test tubes (three tubes per row).
2. Transfer 2 ml of distilled water to tubes in row two for the blank.
3. Transfer 2 ml of diluted sample solutions to screw cap test tubes in triplicate.
4. Add 1 ml of ninhydrin color reagent to all the tubes. Cap tubes with screw caps to avoid evaporation loss.
5. Place the tubes in boiling water bath for exactly 16 minutes.
6. Cool the tubes for 20 minutes in cool water bath (cold tap water is sufficient).

7. Add 5 ml of dilution solution to each tube and mix thoroughly.
8. Measure the absorbance of the solutions at 570 nm against distilled water within 30 minutes of the addition of the dilution solution to the first tube (this is critical as the samples will change color after this time has expired).

Calculation:

Average the absorbance reading obtained from each of the standard, blank, and sample tubes. Subtract the average blank absorbance from the absorbance of each sample and from the glycine standard. Calculate the α-amino nitrogen (αAN) concentration in the sample as follows:

$$\alpha AN = \frac{\text{net absorbance of test solution}}{\text{net absorbance of glycine standard}} \times 2 \times \text{dilution factor}$$

APPENDIX 7: DETERMINATION OF REDUCING SUGARS

Reference:

Miller, G. L., 1959. Anal. Chem. 31:426-428.

Apparatus:

Spectrophotometer, magnetic stirrer with hotplate, water bath, stopwatch, volumetric flasks, beakers, test tubes, pipettes.

Reagents and Solutions:

a) Dinitrosalicylic acid (DNS) reagent: suspend 20.0 g of dinitrosalicylic acid in 400 ml water and gradually add 300 ml of NaOH solution containing 32.0 g NaOH with continuous stirring. Warm the suspension in water bath at 50°C until it is clear. Do not exceed 50°C. Add 600 g potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) gradually to the solution with continuous stirring. Dilute the solution to 2000 ml with deionized water. Store the solution in a dark bottle at room temperature. Reagent is stable for 6 months.
b) Galactose stock solution: dissolve 1.000 g of galactose (dry weight basis; *e.g.*, 2h at 105°C) in deionized water, and bring the volume to 100 ml.
c) Galactose working solutions: dilute 1 ml, 2ml, 4ml, 6 ml, 8 ml, 10 ml and 12 ml of galactose stock solution to 100 ml using deionized water.

Procedure:

1. Standard Curve: pipette 1 ml each of standard working solution into duplicate 10 ml test tubes; add 1 ml deionized water and 3 ml of DNS reagent to each tube. Pipette 2 ml deionized water and 3 ml of DNS reagent into the tubes for reagent blank. Stir the tubes carefully and boil together for exactly 5 min. The water must boil all the time. Stop the color development by cooling the reaction mixtures in an ice-cold water bath for 5 min and allow to equilibrate to room temperature for 20 min. Stir the tubes and measure absorbance at 540 nm using deionized water to set absorbance at zero. Plot the standard curve using absorbance vs. concentration. In order to minimize variation, it is advisable

to construct a standard curve for every series of assays. Also, samples and standard curve should be prepared, boiled, and read at the same time.

2. Measurement:

   a) Pipette 1 ml sample solution into 10 ml test tubes with duplicate.

   b) Add 1 ml distilled water and 3 ml DNS reagent, and mix thoroughly.

   c) Pipette 2 ml deionized water and 3 ml of DNS reagent in the tubes for reagent blank.

   d) Place the tubes in a boiling water bath and boil for exact 5 min.

   e) Cool the tubes in an ice-cold water bath for 5 min.

   f) Equilibrate the tubes at room temperature for 20 min.

   g) Measure the absorbance at 540 nm.

   h) Calculate the concentration of reducing sugars in the sample solution based on the standard curve.

   i) If the absorbance value is beyond the range of the standard curve, dilute the supernatant and redo it again until it is within the range of the standard curve.

APPENDIX 8: DETERMINATION OF TRYPSIN INHIBITOR

Reference:

Kunitz, M., 1947. J. Gen. Physiol. 30:291-310.

Kakade, M. L., N. Simons, and I. E. Liener, 1969. Cereal Chem. 46: 518-526.

Apparatus:

UV spectrophotometer, pH meter, magnetic stirrer with hotplate, water bath, mechanical shaker, stopwatch, volumetric flasks, beakers, test tubes, pipettes and Whatman No. 42 filter paper.

Reagents and Solutions:

a) Phosphate buffer (0.1 M, pH 7.6): dissolve 23.3 g $Na_2HPO_4 \cdot 7H_2O$ and 1.8 g $NaH_2PO_4 \cdot H_2O$ in 900 ml water. Adjust pH to 7.6 and the final volume to 1 L.

b) Casein solution (l or 2%): suspend l or 2 g of casein (Hammersten quality, Nutritional Biochemicals Corp., Cleveland, Ohio) in 80 ml of the phosphate buffer. Heat the solution in a steam bath for 15 min or until casein is completely dissolved. Cool and adjust the solution to 100 ml with buffer, and store in the refrigerator when not in use.

c) Stock trypsin solution: weigh 4 to 5 (±0.0001) mg trypsin (2 x crystallized, salt-free, Worthington Biochemical Corp., Freehold, N.J.) and dissolve it in 100 ml 0.001 M HCl. This solution can be stored in the refrigerator for 2 to 3 weeks without appreciable loss in activity.

d) Preparation of soybean samples: grind mature soybeans to fine particles and extract with petroleum ether at room temperature. Suspend 1 g (±0.0001) of the extracted meal in 19 ml water, and adjust the pH of the suspension to 7.6. After mechanical shaking for 1 hr, centrifuge the suspension and dilute 1 ml of the supernatant to 50 ml with phosphate buffer.

Procedure:

1. Trypsin standard curve: pipette 0.2 to 1.0 ml of the stock trypsin solution into a triplicate set of test tubes (one set for each level of trypsin) and adjust the final volume of each tube

to 2 ml with the phosphate buffer. Place the tubes in a water bath at 37°C. Add 6 ml 5% (w/v) trichloroacetic acid to one of the triplicate tubes; this tube serves as a blank (see below); Add 2 ml of the casein solution, previously brought to 37°C, to each tube. Allow the tubes to remain at 37°C for exactly 20 min. Then stop the reaction by adding 6 ml of 5% trichloroacetic acid to the experimental tubes. Let the tubes stand for 1 hr at room temperature. Filter the suspension, and measure the absorbance of the filtrate at 280 nm against the blank.

2. Trypsin Inhibitor Activity: pipette 0.2 to 1.0 ml aliquots of the soybean extract into a triplicate set of the test tubes (one set for each level of extract), and bring the volume to 1.0 ml with the phosphate buffer. Add 1 ml of the stock trypsin solution to each tube, and place the tubes in the water bath at 37ºC. The remainder of the procedure is the same as that described in the preceding paragraph.

Calculation and Expression of Activity:

One trypsin unit (TU) is arbitrarily defined as an increase of 0.01 absorbance units at 280 nm in 20 min per 10 ml of the reaction mixture under the conditions set forth herein. Trypsin inhibitor activity is defined as the number of trypsin units inhibited (TUI).

APPENDIX 9: NITROGEN DETERMINATION

Reference:

AOAC: Official Methods of Analysis. Association of Official Analytical Chemists, Washington, DC, 1995.

Apparatus:

LECO® FP-2000 Nitrogen/Protein Analyzer (Leco Corporation, Saint Joseph, MI, U.S.A.).

Reagents:

Ethylenediaminetetra-acetic acid (EDTA) (N = 9.59%)

Procedure:

1. Check the gas tanks of O_2 and He; the pressure of the tanks should be more than 200 psi.
2. Open gas and touch screen to log in.
3. Check the leak of O_2 and He for 60 sec. to make sure in good leak.
4. Select ANALYZE button and start analyzing.
5. Analyze at least five blank samples (no boat and no sample) first. The output of the nitrogen percentage should go down until at least three values are very close.
6. Weigh five EDTA standards with about 200 mg for each into sample boat.
7. Recalibrate LECO analyzer for nitrogen analysis using the results of the EDTA standards.
8. Weigh about 400 mg sample into boat.
9. Enter sample weight in computer associated to the LECO Analyzer.
10. Analyze and obtain recorded results for percentage of nitrogen.

Calculations:

Results obtained as % of nitrogen in the sample present in the ceramic boat.

APPENDIX 10: CHROMIUM DETERMINATION (FROM CHROMIC OXIDE)

Reference:

Williams, C. H., D. J. David, and O. Iismaa, 1962. J. Agr. Sci. 59:381.

Apparatus:

Atomic absorption spectrophotometer, muffle furnace, hotplate, volumetric flasks, beakers.

Reagents and Solutions:

a) Acid manganese sulfate: dissolve 2.27 g manganese sulfate ($MnSO_4 \cdot 1H_2O$) to 30 ml deionized water; then carefully add to 970 ml of 85% phosphoric acid.
b) Potassium bromate solution: dissolve 45 g potassium bromate to 800 ml of deionized water; bring the volume to 1 L with water.
c) Calcium chloride solution: dissolve 14.65 g calcium chloride ($CaCl_2 \cdot H_2O$) to 800 ml of deionized water; bring the volume to 1 L with water.

Procedure:

1. Ash approximately 0.5 g of a weighed sample in a crucible at 600ºC overnight.
2. Cool sample and add 3 ml acid manganese sulfate and 4 ml of potassium bromate, being careful not to blow any ash from the crucible.
3. Place on a preheated hot plate and digest ash until effervescence of the solution ceases.
4. Allow crucible to cool, then dilute sample with water and wash completely into tared 100 ml plastic sample cup. Add 12.5 ml calcium solution and bring sample weight up to 100 g.
5. Allow solution to stand overnight to let suspended particles settle.
6. Carefully transfer at least 10 ml aliquots of the supernatant to 15 ml tubes.
7. Read on atomic absorption spectrophotometer following appropriate instrument instruction.

Calculations:

$$\% \text{ Cr} = \frac{\text{Cr, ppm x dilution factor 1 x dilution factor 2 x 0.0001}}{\text{Sample wt, g}}$$

Dilution factor 1 in this procedure is the 100 ml or total volume of final solution analyzed.

Dilution factor 2 is 1 in this procedure because the concentration fits in the standard curve; otherwise, the final solution may require dilution to fall within the linear range of the atomic absorption spectrophotometer.

APPENDIX 11: GROSS ENERGY DETERMINATION

Reference:

AOAC: Official Methods of Analysis. Association of Official Analytical Chemists, Washington, DC, 1995.

Apparatus:

Bomb calorimeter (Part Instrument Company, Moline, IL, U.S.A.)

Reagents:

Benzoic acid, 6318 cal/g

Procedure:

1. Turn on the Bomb calorimeter and select operation.
2. Turn heater and pump and preheat the machine for at least 30 min.
3. Push F_2 button to pretest the cycle.
4. Weigh approximately 1 ± 0.0001 g of benzoic acid (standard reagent) or testing samples into metallic crucible.
5. Carefully place crucible into the crucible holder.
6. Remove any moisture from the heating wire prior to attaching the cotton thread.
7. Install bomb head in calorimeter.
8. Close calorimeter cover.
9. Press START to begin analysis. The first sample should be the standard check sample (benzoic acid). Calorimeter will prompt operator for Sample ID numbers and weights in accordance with operating modes set into instrument.
10. Enter sample ID and sample weight.
11. Ignite.
12. After 6-8 min, the energy value of the sample is printed out in cal/g.

APPENDIX 12: DETERMINATION OF NEUTRAL DETERGENT FIBER

Reference:

Van Soest, P. J., 1967. J. A. O. A. C. 50:50-55.

Apparatus:

ANKOM200/220 Fiber Analyzer (ANKOM Technology, Macedon, NY, U.S.A.). F57 filter Bags (ANKOM Technology, Macedon, NY, U.S.A.), impulse bag sealer and desiccator

Reagents and Solutions:

a) Neutral detergent solution (ND) -- Add 30.0 g sodium lauryl sulfate, USP; 18.61 g ethylenediaminetetraacetic disodium salt, dihydrate; 6.81 g sodium tetraborate decahydrate; 4.56 g sodium phosphate dibasic, anhydrous; and 10.0 ml triethylene glycol, in 1 L distilled H2O. Agitate and heat to facilitate solubility. Check that pH is in 6.9 to 7.1.
b) Alpha-amylase -- *Taka-Therm L-340* (Solvay Enzymes, Inc., Elkart, IN; 800-342-2097): heat stable amylase used for high starch samples (*e.g.*, feed samples). For every load in the ANKOM fiber analyzer, 4 ml of the Taka-Therm L-340 is added to promote starch digestion.
c) Sodium sulfite -- Na_2SO_3, anhydrous.
d) Acetone -- Technical grade that is free from color and leaves no residue upon evaporation.

Procedure:

a) Prepare Sample

    1) Weigh Filter Bag (W1), record weight and tare balance.
    2) Weigh 0.5g (±0.05 g) of air-dried sample (W2), ground to pass through a 1mm screen, directly into filter bag. Weigh one blank bag and include in digestion to determine blank bag correction (C1).
    3) Seal the bags closed within 0.5 cm from the open edge using the heat sealer.

4) Spread sample uniformly inside the filter bag by shaking and lightly flicking the bag to eliminate clumping.
5) A maximum of 24 bags may be placed in the bag suspender. All nine trays are used regardless of the number of bags being processed. Place three bags per tray and then stack trays on center post with each level rotated 120 degrees. A weight is placed on top of the empty 9th tray to keep the bag suspender submerged.
6) NOTE: SAMPLES CONTAINING SOY PRODUCT OR >5% FAT - Extract fat from samples by placing 24 bags with samples into a 500 ml bottle with a top. Pour enough acetone into bottle to cover bags and secure top. Shake the container 10 times and allow bags to soak for 10 minutes. Repeat with fresh acetone. Pour out acetone and place bags on a wire screen to air-dry (approximately 5 minutes).

b) When processing 24 sample bags, add 1900-2000 ml of neutral detergent solution into ANKOM Fiber Analyzer vessel. If processing less than 20 bags, add 100 ml/bag of detergent solution (minimum of 1500 ml). Add 20 g (0.5 g/50 ml of ND solution) of sodium sulfite to the solution in the vessel and 4.0 ml heat stable alpha-amylase.

c) Place bag suspender with samples into the solution in vessel. Turn Agitate and Heat ON and confirm that Bag Suspender is agitating properly. Set timer for 75 minutes and push Start. Close and seal lid of vessel.

d) After 75 minutes (timer will beep), turn Agitate and Heat OFF, open the drain valve and exhaust hot solution before opening lid.

e) After the solution has been exhausted, close valve and open the lid. Add approximately 2000 ml of hot (90-100° C) water and 4.0 ml of alpha-amylase to the first and second rinses. Lower lid but do not tighten. Turn Agitate ON and leave Heat OFF. Each rinse should last 3-5 minutes. Exhaust water and repeat rinse two more times (total of three rinses).

f) Remove filter bags from bag suspender and gently press out excess water. Place in beaker and soak in acetone. Allow bags to soak 3 minutes, then remove and lightly press out excess acetone.

g) Spread bags out and allow acetone to evaporate. Complete drying in oven at 105° C for at least 2 hours. Cool to ambient temperature and weigh bags.

Calculations:

$$\text{NDF, \% (as-is basis)} = \frac{[W_3 - (W_1 \times C)] \times 100}{W_2}$$

W_1 = Bag tare weight.

W_2 = Sample weight.

W_3 = Weight after extraction.

C = Blank bag correction (final oven-dried weight/original blank bag weight).

REFERENCES

Aastrup, S., and L. Muck, 1985. A β-glucan mutant in barley with thin cell wall. Pages 291-296 in New Approach on Cereal Grain Carbohydrate.

Adams, O. L., and E. C. Naber, 1969. Effect of physical and chemical treatment of grains on growth of and feed utilization by the chicks. 1. The effect of water and acid treatments of corn, wheat, barley and expanded or germinated grains on chick performance. Poultry Sci. 48:853-858.

Ademark, P., M. Larsson, F. Tjerneld, and H. Stalbrand, 2001. Multiple α-galactosidases from *Aspergillus niger*: purification, characterization and substrate specifications. Enzyme Microb. Tech. 29:441-448.

Adeola, O., 1995. Digestive utilization of minerals by weanling pigs fed copper- and phytase-supplemented diets. Can. J. Anim. Sci. 75:603-610.

American Soybean Association, 2003. General statistics. Available on the World Wide Web: http://www.asa-europe.org/general.shtml.

Anderson, J. O., D. C. Dobson, and R. K. Wagstaff, 1961. Studies on the value of hulless barley in chick diets and means of increasing this value. Poultry Sci. 40:1571-1584.

Annison, G., and M. Choct, 1991. Antinutritive activities of cereal non-starch polysaccharides in broiler diets and strategies for minimizing their effects. World's Poultry Sci. 47:232–242.

Annison, G., 1993. The role of wheat non-starch polysaccharides in broiler nutrition. Austral. J. Agri. Res. 44:405-422.

Antoniou, T., and R. R. Marquardt, 1981. Influence of rye pentosans on the growth of chicks. Poultry Sci. 60:1898-1904.

AOAC: Official Methods of Analysis. Association of Official Analytical Chemists, Washington, DC, 1995.

Baas, T. C., and P. A. Thacker, 1996. Impact of gastric pH on dietary enzyme activity and survivability in swine fed β-glucanase supplemented diets. Can. J. Anim. Sci. 36:245-252.

Bailey M. J., and K. Poutanen, 1989. Production of xylanases by strains of *Aspergillus*. Appl. Microbiol. Biot. 30:5-10.

Baker, D. H., 1977. Xylose and xylan utilization by the chick. Poultry Sci. 56:2105-2107.

Bamford, C. W., 1982. Barley β-glucan: their role in malting and brewing. Brewer 57:22-35.

Bathgate, G. N., 1979. The determination of endo-glucanase activity in malt. J. I. Brewing. 85:92-94.

Beal, J. D., P. H. Brook, and H. Schulze, 1998a. The effect of pretreatment with different proteases on the *in vitro* digestibility of nitrogen in raw soy bean and four different full fat soy bean meals. Page 264 in Book of Abstracts of the 49th Annual Meeting of the European Association for Animal Production, Warsaw, Poland. van Arendonk, J. A. M., ed. Wageninggen Pers, Wageningen, The Netherlands.

Beal, J.D., P.H. B rooks, and H. Schulze, 1998b. The hydrolysation of protein in raw and autoclaved soybean meals by a microbial protease. Page 167 in British Society of Animal Science Winter Meeting, Scarborough, UK.

Beauchemin, K. A., D. Colombatto, D. P. Morgavi, and W. Z. Yang, 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. J. Anim. Sci. 81:E37-E47.

Bedford, M. R., H. L. Classen, and G. L. Campbell, 1991. The effect of pelleting, salt, and pentosanase on the viscosity of intestinal contents and the performance of broilers fed rye. Poultry Sci. 70:1571-1577.

Bedford, M. R., and H. Schulze, 1998. Exogenous enzymes for pigs and Poultry (Review). Nutr. Res. Rev. 11:91-114.

Berchieri, A. JR., and P. A. Barrow, 1996. Reduction in incidence of experimental fowl typhoid by incorporation of a commercial formic acid preparation (Bio-AddTM) into Poultry feed. Poultry Sci. 75:339-341.

Berg, L. R., 1961, Effect of adding enzyme to barley diets at different ages of pullet on laying house performance. Poultry Sci. 40:34-39.

Boling, S. D., C. M. Parsons, and D. H. Baker, 1999. Citric acid improves phytate phosphorus utilization in broiler chicks fed corn-soybean meal diets. Poultry Sci. 78 (Suppl. 1): S31(Abs.).

Boling, S. D., D. M. Webel, I. Mavromichalis, C. M. Parsons, and D. H. Baker, 2000. The effects of citric acid on phytate-phosphorus utilization in young chicks and pigs. J. Anim. Sci. 78:682-689.

Bolton, W., and Dewar, 1964. The digestibility of acetic, propionic and butyric acids by the fowl. Br. Poult. Sci. 6:103-105.

Bolton, W., 1965. Digestion in the crop of the fowl. Br. Poult. Sci. 6:97-102.

Brenes, A., M. Smith, W. Guenter, and R. R. Marquardt, 1993. Effect of enzyme supplementation on the performance and digestive tract size of broiler chickens fed wheat and barley based diets. Poultry Sci. 72:1731-1739.

Broek, G. V. D., M. V. D. Bergh, B. Ebbinge, B. V. Selko, and Tilburg, 2003. Clean drinking water during production by use of organic acids. World Poultry. 19:34-37.

Brown, D. R., and L. Lee Southern, 1985. Effect of citric and ascorbic acids on performance and intestinal pH of chicks. Poultry Sci. 64:1399-1401.

Burnett, G. S., 1966. The effect of damaged starch, amylolytic enzymes, and proteolytic enzymes on the utilization of cereals by chickens. Br. Poult. Sci. 3:89-103.

Campbell, G. L., F. W. Sosulski, H. L. Classen, and G. M. Balance, 1986b. The nutritive value of irradiated and β-glucanase-treated wild oat groats for broiler chickens. Anim. Feed Sci. and Tech. 16:143-252.

Campbell, G. L., and A. F. B. van der Poel, 1998. Use of enzymes and process technology to inactivate antinutritional factors in legume seeds and rapeseed. Pages 377-386 in Proc. Recent Advances of Research in Antinutritional Factors in Legume Seeds and Rapeseed. Wageningen Press, Wageningen, The Netherlands.

Cantor, A. H., A. J. Pesctore, T. H. Johnson, and W. K. Pfaff, 1989. Influence of β-glucanase Allzyme on performance of broiler chicks fed barley-based diets. Pages 161-166 in Biotechnology in the Feed Industry: Proc. Alltech's 5th Annual Symposium. T. P. Lyon, ed. Alltech Technical Publication, Nicholasville, KY.

Cave, N. A. G., 1982. Effect of dietary short-chain fatty acids on feed intake by chicks. Poultry Sci. 61:1147-1153.

Cave, N. A. G., 1983. Glycine- and fatty-acid-induced restriction of feed intake: effects on body weights and hatching egg production of broiler breeders restricted from day of hatching. Poultry Sci. 62:125-132.

Cave, N. A. G., 1984. Effect of dietary propionic and lactic acids on feed intake by chicks. Poultry Sci. 63:131-134.

Chazi, S., J. A. Rooke, and H. Galbraith, 2003. Improvement of the nutritive value of soybean meal by protease and alpha-galactosidase treatment in broiler cockerels and broiler chicks. Br. Poult. Sci. 44(3):410-8.

Cherrington, C. A., M. Hinton, G. C. Mead, and I. Chopra, 1991. Organic acids: chemistry, antibacterial activity and practical applications. Adv. Microb. Physiol. 32:87-107.

Chesson, A., 1987. Supplementary enzymes to improve the utilization of pig and poultry diets. Pages 71-89 in Recent Advances in Animal Nutrition. Haresign, W. and Cole, D. J. A., ed. Butterworths, UK.

Chesson, A., 1993. Feed enzymes. Anim. Feed Sci. and Tech. 45:65-79.

Choct, M., and G. Annison, 1990. Anti-nutritive activity of wheat pentosans in poultry diets. Br. Poult. Sci. 31:809-819.

Choct, M., R. J. Hughes, J. Wang, M. R. Bedford, A. J. Morgan, and G. Annison, 1996. Increased small intestine fermentation is partly responsible for the anti-nutritive activity of non-starch polysaccharides in chickens. Br. Poult. Sci. 37:609-621.

Clarkson, K., B. Jones, R. Bott, B. Bower, G. Chotani, and T. Becker, 2001. Enzymes: screening, expression, design and production. Pages 315-352 in Enzymes in Farm Animal Nutrition. M. R. Bedford and G. G. Partridge, ed. UK.

Classen, H. L., and G. I. Campbell, 1990. Improvement of feed utilization through the use of enzyme products. Proceedings of the Australian Poultry Science Symposium 1-8.

Classen, H. L., and M. R. Bedford, 1991. The use of enzymes to improve the nutritive value of poultry feeds. Pages 95-115 in Recent Advances in Animal Nutrition . W. Haresign and D. J. A. Cole, ed. Butterworth-Heinemann, London, U.K.

Classen, H. L., D. Balnave, and M. R. Bedford, 1993. Reduction of legume antinutritional factors using biotechnological techniques. Pages 501- 516 in Recent Advances of Research in Antinutritional Factors in Legume Seeds. Proceedings of the Second International Workshop on 'Antinutritional factors (ANFs) in Legume seeds'. van der Poel, A.F.B., Huisman, J. and Saini, H.S., ed. Wageningen Pers, Wageningen, The Netherlands.

Coon, C. N., K. L. Leske, O. Akavanichan, and T. K. Cheng, 1990. Effect of oligosaccharide-free soybean meal on true metabolizable energy and fiber digestion in adult roosters. Poultry Sci. 69:787-793.

Coughlan, M. P., 1985. The properties of fungal and bacterial cellulases with comment on their production and application. Biotachnol. Genet. Eng. Rev. 3:39-109.

Cowan, D., 1992. Advances in feed enzyme technology. Agro. Food Industry Hi. Tech. 3(3), 9.

CVB (Centraal Veevoederbureau), 1998. Table of feedstuffs. Information about composition, digestibility and feeding value. Centraal Veevoederbureau, Lelystad, The Netherlands.

Danicke, S., G. Dusel, H. Jeroch, and H. Kluge, 1999. Factors affecting efficiency of NSP-degrading enzymes in rations for pigs and poultry. Agribiolog. Res. 52:1-24.

Dierick, N., and J. Decuypere, 1996. Mode of action of exogenous enzymes in growing pig nutrition. Pig News Information 17:41N-48N.

Dudley-Cash, W. A., 2001. Soybean meal source, added enzyme affect nutritional value for chicks. Feedstuffs 73:41.

Duncan, M. S., and A. W. Adams, 1972. Effects of chemical additive and of formaldehyde-gas fumigation on *Salmonella* in poultry feeds. Poultry Sci. 51:797-802.

Edwards, C. A., I. T. Jonhnson, and N. W. Read, 1988. Do viscous polysaccharides slow absorption by inhibiting diffusion or convection? European J. Clinical Nutr. 42:306-312.

Edwards, H. M. Ш, and D. H. Baker, 1999. Effect of dietary citric acid on zinc bioavailability from soy products using an egg white diet with zinc sulfate heptahydrate as the standard. Poultry Sci. 78 (Suppl. 1):576 (Abstr.).

ESC, 2004. Enzyme Services & Consultancy at www.enzymes.co.uk.

Falkowski, J. F., and F. X. Aherne, 1984. Fumaric and citric acid as feed additives in starter pig nutrition. J. Anim. Sci. 58:935-938. Food Chemical Codex WA 701 N277f, 1996 page 813.

Farrand, E. A., 1964. Flour properties in relation to the modern bread process in the United Kingdom with specific reference to alpha-amylase and starch damage. Cereal Chem. 41:98-111.

FCC: Food Chemicals Codex , WA 701 N277f, 1996

Fengler, A. I., and R. R. Marquardt, 1988. Water-soluble pentosans from rye: П. Effects on rate of dialysis and on the retention of nutrients by the chick. Cereal Chem. 65: 298-302.

Friesen, O. D., W. Guenter, B. A. Rotter, and R. R. Marquardt, 1991. The effects of enzyme supplementation on the nutritive value of rye grain for the young broiler chick. Poultry Sci. 70:2501-2508.

Fry, R. E., J. B. Allred, L. S. Jensen, and J. McGinnis, 1957. Influence of water treatment on nutritive value of barley. Proc. Soc. Exp. Biol. Med. 95:249-251.

Fry, R. E., J. B. Allred, L. S. Jensen, and J. McGinnis, 1958. Influence of enzyme supplementation and water treatment on the nutritional value of different grains for poults. Poultry Sci. 37:372-375.

Gabert, V. M. and W. C. Sauer. 1994. The effects of supplementing diets for weanling pigs with organic acids. A review. J. Anim. Feed Sci. 3:73-87.

Gdala, G., A. J. M. Jansman, L. Buraczewska, J. Huisman, and P. van Leeuwen, 1997. The influence of a-galactosidase supplementation on the ileal digestibility of lupin seed carbohydrates and dietary protein in young pigs. Anim. Feed Sci. Tech. 67:115-125.

Ghazi, S., J. A. Rooke, H. Galbraith, and M. R. Bedford, 2002. The potential for the improvement of the nutritive value of soybean meal by different proteases in broiler chicks and broiler cockerels. Br. Poult. Sci. 43(1):70-7.

Ghazi S., J. A. Rooke, and H. Galbraith, 2003. Improvement of the nutritive value of soybean meal by protease and α-galactosidase treatment in broiler cockerels and broiler chicks. Br. Poult. Sci. 44(3):410-8.

Giesting, D. W., and R. A. Easter, 1985. Response of starter pigs to supplementation of corn-soybean meal diets with organic acids. J. Anim. Sci. 60:1288-1294.

Gitzelmann, R., and S. Auricchio, 1965. The handling of soy α-galactosidase by a normal and galactosemic child. Pediatrics 36:231-232.

Gohl, B., S. Alden, K. Elwinger, and S. Thomke, 1978. Influence of β-glucanase on feeding value of barley for poultry and moisture content of excreta. Br. Poult. Sci. 19: 41-47.

Gomez, S., S. E. Scheideler, and C. Wyatt, 1999. Effects of enzyme supplementation to corn-soybean rations with different energy densities on laying hen performance. Poultry Sci. (supplement) 78:103.

Gordon, D. T., 1990. Total dietary fiber and mineral absorption. In: Dietary Fiber (D. Kritchevsky, C. Bonfield, and J. W. Anderson, ed.) pp. 105-128. Plenum Press, NY.

Gracia, M. I., M. J. Aranibar, R. Lazaro, P. Medel, and G. G. Mateos, 2003. α-amylase supplementation of broiler diets based on corn. Poultry Sci. 82:436-442.

Graham, K. K., M. S. Kerley, J. D. Firman, and G. L. Allee, 2002. The effect of enzyme treatment of soybean meal on oligosaccharide disappearance and chick growth performance. Poultry Sci. 81:1014-1019.

Graf, E., 1986. Chemistry and applications of phytic acid: an overview. Pages 173-194 in Phytic Acid: chemistry and applications. E. Graf, ed. Pilatus Press, Minneapolis.

Grootwassink, J. W. D., G. L. Campbell, and H. L. Classen, 1989. Fractionation of crude pentosanase for improvement of the nutritional value of rye diets for broiler chickens. J. Sci. Food Agric. 46:289-300.

Hastings, W. H., 1946. Enzyme supplements to poultry feeds. Poultry Sci. 25:548-586.

Herpol, C., and G. Van Grembergen, 1967. La significance du pH dans le tube digetif de gallus domesticus. Pages 75-79 in Comparative Nutrition of Fowl and Swine: The Gastrointestinal Systems. E. T. Moran, Jr., ed. Canada.

Hesselman, K., K. Elwinger, M. Nilson, and S. Thomke, 1981. The effect of β-glucanase supplementation, stage of ripeness, and storage treatment of barley diets fed to broiler chickens. Poultry Sci. 60:2664-2671.

Hesselman, K., and P. Aman, 1986. The effect of β-glucanase on utilization of starch and nitrogen by broiler chickens fed on barley of low and high viscosity. Anim. Feed Sci. Tech. 15:83-93.

Heuser, G. F., 1945. The rate of passage of feed from the crop of the hen. Poultry Sci. 24:20-24.

Hinton, M., A. H. Linton, and F. G. Perry, 1985. Control of S*almonella* by acid disinfection of chick's food. Vet. Rec. 116:(8):502.

Hinton, M., and A. H. Linton, 1988. Control of *Salmonella* infection in broiler chickens by the acid treatment of their feed. Vet. Rec. 123:416-421.

Hinton, A. JR., R. J. Buhr and K. D. Ingram, 2000. Reduction of *Salmonella* in the crop of broiler chickens subjected to feed withdrawal. Poultry Sci. 79:1566-1570.

Hoopaw, P. D., and B. L. Goodman, 1976. The influence of intermittent light on growth and performance and other traits in young chicks. Poultry Sci. 55:2285.

Huisman, J., and A. J. M. Jansman, 1991. Dietary effects and analytical aspects of antinutritional factors in peas and soybeans in monogastric farm animals. A literature review. Nutr. Abs. and Rev., 61B: 901-921.

Huisman, J., and G. H. Tolman, 1992. Antinutritional factors in the plant protein of diets for non-ruminants. Recent Advances in Animal Nutrition. Butterworth-Heinemann, Oxford, pp. 3-32.

Hume, M. E., D. E. Corrier, G. W. Ivie, and J. R. Deloach, 1993. Metabolism of [^{14}C] propionic acid in broiler chicks. Poultry Sci. 72:786-793.

Humphrey, T. J., and D. G. Lanning, 1988. The vertical transmission of *Salmonellas* and formic acid treatment of chicken feed. A possible strategy for control. Epidemiol. Infect. 100:43-49.

Huo, G.C., V.R. Fowler, and M. Bedford, 1993. The use of enzymes to denature antinutritive factors in soybean. Pages 517- 521 in Recent Advances of Research in Antinutritional Factors in Legume Seeds. Proceedings of the Second International Workshop on 'Antinutritional factors (ANFs) in Legume seeds'. van der Poel, A.F.B., Huisman, J. and Saini, H.S., ed. Wageningen Pers, Wageningen, The Netherlands.

Iba, A. M., and A. JR. Berchieri, 1995. Studies on the use of a formic acid-propionic acid mixture (Bio-add^{TM}) to control experimental *Salmonella* infection in broiler chickens. Avian Pathol. 24:303-331.

Ikegami, S., F. Tsuchihashi, H. Harada, N. Tsuchihashi, E. Nishide, and S. Innami, 1990. Effect of viscous indigestible polysaccharides on pancreatic-biliary secretion and digestive organs in rats. J. Nutr. 120:353-360.

Irish, G. G., and D. Balnave, 1993. Poor performance of broilers fed diets containing soybean meal as the sole protein concentrate. Austra. J. Agri. Res. 44:1467-1481.

Irish, G. G. and D. Balnave, H. L. Classen, R. T. Tyler, and M. R. Bedfrod, 1995. Removal of the α-galactosides of sucrose from soybean meal using either ethanol extraction or exogenous α-galactosidase and broiler performance. Poultry Sci. 74:1484-1494.

Izat, A. L., N. M. Tidwell, R. A. Thomas, M. A. Reiber, M. H. Adams, M. Colberg, and P. W. Waldroup, 1990. Effects of a buffered propionic acid in diets on the performance of broiler chickens and on microflora of the intestine and carcass. Poultry Sci. 69:818-826.

Izat, A. L., N., M. H. Adams, M. C. Cabel, M. Colberg, M. A. Reiber J. T. Skinner, and P. W. Waldroup, 1990. Effects of formic acid or calcium formate in feed on performance and microbiological characteristics of broilers. Poultry Sci. 69:1876-1882.

Jayne-Williams, D. J., and R. Fuller, 1971. The influence of the intestinal microflora on nutrition. In Physiology and Biochemistry of the Domestic Fowl Vol. 1. ed. By D. J. Bell and B. M. Freeman. Academic Press, London.

Jensen, L. S., R. E. Fry, J. B. Allred, and J. McGinnis, 1957. Improvement in the nutritional value of barley for chicks by enzyme supplementation. Poultry Sci. 36:919-921.

Johnson, I. T., and M. G. Jennifer, 1981. Effect of gel-forming gums on the intestinal unstirred layer and sugar transport in vitro. Gut 22:398-403.

Kakade, M. L., N. Simons, and I. E. Liener, 1969. An evaluation of natural vs. synthetic substrates for measuring the antitryptic activity of soybean samples. Cereal Chem. 46: 518-526.

Kerley, M. S., and G. L. Allee, 2003. Modification in soybean seed composition to enhance animal feed use and value; moving from a dietary ingredient to a functional dietary component. Ag. Bio. forum 6(1&2):14-17.

Kidd, M. T., G. W. Morgan, and C. D. Zumwalt, 2001. α-galactosidase enzyme supplementation to corn and soybean meal broiler diets. J. Appl. Poult. Res. 10:186-193.

Kirchgessner, M., and F. X. Roth, 1982. Fumaric acid as a feed additive in pig nutrition. Pig News Info. 3:259-264.

Kirchgessner, M., and F. X. Roth, 1991. Ergopic effects through the nutritive use of organic acids. Zentralbl Hyg Umweltmed 191(2-3):265-76.

Kocher, A., M. Choct, M. D. Porter, and J. Broz, 2002. Effect of feed enzymes on nutritive value of soybean meal fed to broilers. Br. Poult. Sci. 43(1):54-63.

Kocher, A., M. Choct, G. Ross, J. Broz, and T. K. Chung, 2003. Effects of enzyme combinations on apparent metabolizable energy of corn-soybean meal-based diets in broilers. J. Appl. Poult. Res. 12:275-283.

Kornegay, E. T., 2001. Digestion of phosphorus and other nutrients: the role of phytases and factors influencing their activity. In: M. R. Bedford and G. G. Partridge. Enzymes in Farm Animal Nutrititon. CABI Publishing, pp. 237-271.

Kunitz, M., 1947. Crystalline soybean trypsin inhibitor. II. General properties. J. Gen. Physiol. 30:291-310.

Kuriyama, S., and L. B. Mendel, 1917. The physiological behavior of raffinose. J. Biol. Chem. 31:125-147.

Lalles, J. P., 1993. Nutritional and antinutritional aspects of soybean and field pea proteins used in veal calf production: a review. Livest. Prod. Sci. 34:181-202.

Langhout, P., 2000. New additives for broiler chickens. FEED MIX Special: Alternatives to antibiotics. pp 24-27.

Lesher, S., H. E. Wallbury, and G. A. Sacher, 1964. Generation cycle in the duodenal crypt cells of germ-free and conventional mice. Nature 202:884-886.

Leske, K. L., O. Akavanichan, T. K. Cheng, and C. N. Coon, 1991. Effect of ethanol extract on nitrogen-corrected true metabolizable energy for soybean meal with broilers and roosters. Poultry Sci. 70:892-895.

Leske, K. L., C. J. Jeven, and C. N. Coon, 1993. Effect of oligosaccharide additions on nitrogen corrected metabolizable energy of soy protein concentrate. Poultry Sci. 72:664-668.

Li, D. F., X. R. Che, Y. Q. Wang, C. Hong, and P. A. Thacker, 1998. The effect of microbial phytase, vitamin D_3, and citric acid on growth performance and phosphorus, nitrogen, and calcium digestibility in growing swine. Anim. Feed Sci. Tech. 73:173-186.

Low, A. G., 1989. Secretory response of the pig gut to non-starch polysaccharides. Anim. Feed Sci. Tech. 23:55-65.

MacAuliffe, T., and J. McGinnis, 1971. Effect of antibiotic supplement to diets containing rye on chick growth. Poultry Sci. 50: 1130-1134.

MacAuliffe, T., A. Pietraszek, and J. McGinnis, 1976. Variable rachitogenic effects of grain and alleviation by extraction or supplementation with vitamine D, fat and antibiotics. Poultry Sci. 55: 2142-2147.

Marsman, G. J. P., H. Gruppen, A. F. B. Van Der Poel, R. P. Kwakkel, M. W. A. Verstegen, and A. G. J. Voragen, 1997. The effect of thermal processing and enzyme treatments of soybean meal on growth performance, illeal nutrient digestibility, and chime characteristics in broiler chicks. Poultry Sci. 76:864-872.

Martin, W. A. V., and B. A. Williams, 2002. Alternatives to the use of antibiotics as growth promoters for monogastric animals. Animal Biotech. 13 (1):97-112.

McGinnis, J., 1983. Carbohydrate utilization in feedstuffs. Pages 106-107 in Proc., Minn. Nutr. Conf., St. Paul, MN 55108.

McClear, B. V., and M. Glennie-Holmes, 1985. Enzymatic quantification of (1-3) (1-4) beta-glucan in barley and malt. J. Inst. Brew. 91:285-295.

Miller, G. L., 1959. Use of dinitrosalisalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31:426-428.

Misir, R., and R. R. Marquardt, 1978. Factors affecting rye utilization in growing chicks. I. The influence of rye level, ergot and penicillin supplementation. Can. J. Anim. Sci. 58: 691-701.

Moran, E. T., I. Martinez-Munoz, and R. C. Hoseney, 1969. The feeding value of rye for growing chick: Effect of enzyme supplements, antibiotics, autoclaving and geographical area of production. Poultry Sci. 48: 939-949.

Moore, S., and W. Stein, 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. 211(2):907-13.

Moran, E. T. Jr., 1982. The Gastrointestinal Systems. Page 74 in Comparative Nutrition of Fowl and Swine, Canada.

Mroz, Z., A. W. Jongbloed, and P. A. Kemme, 1994. Apparent digestibility and retention of nutrients bound to phytate complexes as influenced by microbial phytase and feeding regime in pigs. J. of Anim. Sci. 72:126-132.

National Research Council, 1994. Nutrient Requirements of Poultry. 9th rev. ed. National Academy Press, Washington, DC.

National Research Council, 1998. Nutrient Requirements of Swine. 10th rev. ed. National Academy Press, Washington, DC.

Nelson, T. S., 1967. The utilization of phytate phosphorus by poultry – A review. Poultry Sci. 46:862-869.

Noy, Y., and D. Sklan, 1995. Digestion and absorption in the young chick. Poultry Sci. 74:366-373.

Nursey, I., 1997. Control of Salmonella. Kraftfutter 10:415-22.

Ogden, J. M., 1995. Feeding trial to evaluate the effects of in-feed enzyme inclusion upon growth, food conversion ratio and carcass composition of pigs. MSc thesis, University of Bristal, UK.

Osborne, D. L., and E. R. Seidel, 1989. Microflora derived polymines modulate obstruction induced colonic mucosal hypertrophy. Am. J. Phys. 256:G1049-G1057.

Pallauf, J., and G. Rimbach, 1997. Effect of supplemental phytase on mineral and trace element bioavailability and heavy metal accumulation in pigs with different type diets. In: Phytase in animal nutrition and animal waste management. M. B. Coehlbo and E. T. Kornegay, Ed. BASF publication DC9601.

Parkany-Gyarfas, A., 1975. Utilization of alpha-amylase-supplemented diet in turkey feeding tests. Acta Aliment 4:23-35.

Parsons, C. M., Y. Zhang, and M. Araba, 2000. Nutritional evaluation of soybean meals varying in oligosaccharide content. Poultry Sci. 79:1127-1131.

Partanen, K. H. and Z. Mroz, 1999. Organic acids for performance enhancement in pig diets. Nutr. Res. Rev. 12: 1-30.

Partridge, G. G., 2001. The role and efficacy of carbohydrase enzymes in pig nutrition. Pages 1-10 in Enzymes in Farm Animal Nutrition. M. R. Bedford and G. G. Partridge, ed. CABI Publishing, UK.

Patten, J. D., and P. W. Waldroup, 1988. Use of organic acids in broiler diets. Poultry Sci. 67:1178-1182.

Pettersson, D., and P. Aman, 1988. Effects of enzyme supplementation of Poultry diet containing rye and wheat. Br. J. Nutr. 62:139-149.

Pierson, E. E. M.., L. M. Potter, and R. D. Brown, 1980. Amino acid digestibility of dehulled soybean meal by adult turkeys. Poultry Sci. 59:845-848.

Pileggi, V. J., H. F. De Luca, J. W. Cramer, and H. Steenbock, 1956. Citrate in the prevention of rickets in rats. Arch. Biochem. Biophys. 60:52-57.

Potter, L. M., M. W. Stutz, and L. D. Matterson, 1965. Metabolizable energy anddigestibility coefficients of barley for chicks as influenced by water treatment or by presence of fungal enzyme. Poultry Sci. 44:565-573.

Rackis, J. J., D. J. Seassa, and F. R. Steggerda, 1970. Flavor and flatulence factors in soybean protein products. J. Agric. Food Chem. 18:977-982.

Radcliffe, J. S., Z. Zhang, and E. T. Kornegay, 1998. The effect of microbial phytase, citric acid, and their interaction in a corn-soybean meal-based diet for wealing pigs. J. Anim. Sci. 76:1880-1886.

Radcliffe, J., 2000. British supermarkets: forging changes in Poultry nutrition. Austra. Poult. Sci. Symposium 12:25-31.

Ratto, M., and K. Poutanen, 1988. Production of mannan-degrading enzymes. Biotachnol. Lett. 10:661-664.

Ravindran, V., W. L. Bryden, and E. T. Kornegay, 1995. Phytates: occurrence, bioavailability and implications in Poultry nutrition. Poult. Avian Biol. Rev. 6:125-143.

Ricks, E. L., D. A. Moscatelli, and W. H. Ott, 1962. The isolation and biological properties of a β-glucanase from B. subtilis. Arch. Biochem. Biophys. 96:371-375.

Riley, W. W., and R. E. Austic, 1984. Influence of dietary electrolytes on digestive tract pH and acid-base status of chicks. Poultry Sci. 63:2247-2251.

Ritz, C. W., R. M. Hulet, B. B. Self, and D. M. Denbow, 1995. Growth and intestinal morphology of male turkeys as influenced by dietary supplementation of amylase and xylanase. Poultry Sci. 74:1329-1334.

Rogel, A. M., E. F. Annison, W. L. Bryden, and D. Balnave, 1987. The digestion ofwheat starch in broiler chickens. Aust. J. Agric. Res. 38: 639-649.

Rooke, J. A., H. Fraser, M. Shanks, and A. Morgan, 1996. The potential for improving soya-bean meal in diets for weaned piglets by protease treatment: comparison with other protein sources. British Society of Animal Science Winter Meeting. Scarborough, UK, Paper 136.

Rooke, J.A., M. Slessor, H. Fraser, and J.R. Thomson, 1998. Growth performance and gut function of piglets weaned at four weeks of age and fed protease-treated soybean meal. Anim. Feed Sci. and Tech. 70:175-190.

Roth, F. X., and M. Kirchgessner, 1998. Organic acids as feed additives for young pigs: nutritional nad gastrointestinal effects. J. Anim. Feed Sci. 7:25-33.

Rotter, B. A., R. R. Marquardt, and W. Guenter, 1989a. Enzymes in feed – they really can be made to work. Feed Compounder 9, 32-35.

Rotter, B. A., R. R. Marquardt, W. Guenter, C. Biliaderis, and C. W. Newman, 1989b. *in vitro* viscosity measurements of barley extracts as predictors of growth responses in chicks fed barley-based diets supplemented with a fungal enzyme preparation. Can. J. Anim. Sci. 69:431-439.

Rouse, J., A. Rolow, and C. E. Nelson, 1988. Research Note: Effect of chemical treatment of Poultry feed on survival of Salmonella. Poultry Sci. 67:1225-1228.

Russell, J. B., 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. J. Appl. Bacteriol. 73:363-370.

Sandsted, R. M., E. Kneen, and M. J. A. Blish, 1939. A standardized Wohlgemuth procedure for alpha-amylase activity. Cereal Chemi. 16: 712-723.

Schang, M. J., J. O. Azcona, and J. E. Arias, 1997. Effects of a soya enzyme supplement on performance of broilers fed corn/soy or corn/soy/full-fat soy diets. Poultry Sci. 76 (Suppl. 1), 132 (abstract).

Scheideler, S. E., A. Abudabos, and C. Wyatt, 1999. Effects of enzyme supplementation of corn-soy based layer diet. Poultry Sci. (supplement) 77:55.

Scheideler, S. E., and T. Weber, 2003a. Role of alpha-galactosidase in corn-soy based layer rations. Pages 20-22 in The Nebraska Poult. Report.

Scheideler, S. E., and T. Weber, 2003b. Supplementation of pullet and layer diets with a multi-enzyme product. Pages 27-27 in The Nebraska Poult. Report.

Sebastian, S., S. P. Touchburn, E. R. Chavez, and P. C. Lague, 1996a. The effects of supplemental microbile phytase on the performance and utilization of dietary calcium, phosphorus, copper and zinc in broiler chickens fed corn-soybean diets. Poultry Sci. 75:729-736.

Sebastian, S., S. P. Touchburn, E. R. Chavez, and P. C. Lague, 1996b. Efficacy of supplemental microbial phytase at different calcium levels on growth performance and mineral utilization of broiler chickens. Poultry Sci. 75:1516-1523.

Sebastian, S., S. P. Touchburn, E. R. Chavez, and P. C. Lague, 1997. Apparent digestibility of protein and amino acids in broiler chickens fed a corn-soybean diet supplemented with microbial phytase. Poultry Sci. 76:1760-1769.

Sheppy, C., 2001. The current feed enzyme market and likely trents. In: M. R. Bedford and G. G. Partridge. Enzymes in Farm Animal Nutrititon. CABI Publishing, pp. 1- 10.

Shieh, T. R., R. J. Wodzinski, and J. H. Ware, 1969. Regulation of the formation of acid phosphatases by inorganic phosphate in *Aspergillus ficuum*. J. Bacteriol. 100:1161.

Shohl, A. T., 1937. The effect of the acid-base content of the diet upon the production and cure of rickets with special reference to citrates. J. Nutri. 14:69-83.

Simon, O., 1998. The mode of action of NSP hydrolyzing enzymes in the gastrointestinal tract. J. Anim. Feed Sci. 7:115-123.

Simons, P. C. W., H. A. J. Vesteegh, A. W. Jongbloed, P. A. Kemme, P. Slump, K. E. Bos, M. G. E. Wolters, R. F. Beudeker, and G. J. Verschoor, 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. Br. J. Nutr. 64:525-540.

Skinner, J. T., A. L. Izat, and P. W. Waldroup, 1991. Research notes: Fumaric acid enhances performance of broiler chickens. Poultry Sci. 70:1444-1447.

Smith, H. W., 1965. The development of the flora of the alimentary tract in young animals. J. Path. Bact. 90:495.

Smyser, C. F., and G. H. Snoeyenbos, 1979. Evaluation of organic acids and other compounds as *Salmonella* antagonists in meat and bone meal. Poultry Sci. 58:50-54.

Snedector, G. W., and W. G. Cochran, 1989. Statistical Methods. 8th ed. Iowa State University Press, Ames, IA.

Sonnenwirth, A., and L. Jarett, 1980. Clinical Laboratory Methods and Diagnosis. St. Louis, MO: C.V. Mosby Co. pp 149-92.

Steggerda, F. R., 1968. Gastrointestinal gas following food consumption. Ann. N.Y. Acad. Sci. 150:57-66.

Swift, M. L., M. A. G. van Keyserlingk, A. Leslie, and D. Teltge, 1996. The effect of Allzyme Vegpro supplementation and expander processing on the nutrient digestibility and growth of broilers. 12th Annual Symposium on Biotechnology in the Feed Industry. Lexington, Kentucky. Supplement 1, enclosure code UL 2.1.

Tervila-Wilo, A., T. Parkkonen, A. Morgan, M. Hopeakoski-Nurminen, K. Poutanen, P. Heikkinen, and K. Autio, 1996. *In vitro* digestion of wheat microstructure with xylanase and cellulase from *trichoderma reesei*. J. Cereal Sci. 24:215-225.

Thacker, P. A., and T. C. Bass, 1996. Effects of gastric pH on the activity of exogenous pentosanase and the effect of pentosanase supplementation of the diet on the performance of growing-finishing pigs. Anim. Feed Sci. Technol. 63:187-200.

Thompson, L. U., 1986. Phytic acid: a factor influencing starch digestibility and blood glucose response. Pages 173-194 in Phytic Acid: chemistry and applications. E. Graf, editor. Pilatus Press, Minneapolis.

Thompson, J. L., and M. Hinton, 1997. Antibacterial activity of formic and propionic acids in the diets of hens on salmonellas in the crop. Br. Poult. Sci. 38 (1): 59- 65.

Thorpe, J., and J. D. Beal, 2001. Vegetable protein meals and the effect of enzymes. Pages 1-10 in Enzymes in Farm Animal Nutrition. M. R. Bedford and G. G. Partridge, ed. UK.

Trugo, L. C., A. Farah, and L. Cabral, 1995. Oligosaccharide distribution in Brazilian soya been cultivars. Food Chem. 52:385-387.

Uni, Z., Y. Noy, and D. Sklan, 1995. Posthatch changes in morphology and function of the small intestines in heavy and light strain chicks. Poultry Sci. 74:1622-1629.

Van der wal, P., 1979. *Salmonella* control of feedstuffs by pelleting or acid treatment. Zootechnica (Nov.): 28-32.

Van Soest, P. J., 1967. Use of detergents in the analysis of fibrous feeds. IV. Determination of plant cell-wall constituents. J. A. O. A. C. 50:50-55.

Veldman, A. V., W. A. G. Veen, D. Barug, and P. A. van Paridon, 1993. Effect of a-galactosidase in feed on ileal piglet digestive physiology. J. Anim. Physiol. Anim. Nutr. 69:57-65.

Verstegen, M. W. A., and B. A. Williams, 2002. Alternatives to the use of antibiotics as growth promoters fro monogastric acimals. Anim. Biotech. 13(1):97-112.

Vogt, H., S. Matthes, S. Harnisch, and M. Ristic, 1979. Fumaric acid in broiler rations. Arch. Geflugelkd 43:54-60.

Vogt, H., S. Matthes, and S. Harnisch, 1981. The effect of organic acids in the rations on the performance of broilers and laying hens. Arch. Geflugelkd 45:221-232.

Watkins, S., 2004. Effects of water acidification on broiler performance. Avian Advice 6:2.

White, W. B., H. R. Bird, M. L. Sunde, J. A. Marlet, N. A. Prentice, and W. C. Burger, and J. A. Marlet, 1981. The viscosity interaction of barley β-glucan with *Trichoderma viride* cellulose in the chick intestine. Poultry Sci. 60:1043-1048.

White, W. B., H. R. Bird, M. L. Sunde, J. A. Marlet, N. A. Prentice, and W. C. Burger, 1983. Viscosity of β-D-glucan as a factor in the enzymic improvement of barley for chicks. Poultry Sci. 62: 853-862.

Wiggins, H. S., 1984. Nutritional value of sugars and related compounds undigested in the small gut. Proc. Nutr. Soc. 43:69-85.

William W. Riley, JR, and Richard E. Austic, 1984. Influence of dietary electrolytes on digestive tract pH and acid-base status of chicks. Poultry Sci. 63:2247- 2251.

Williams, C. H., D. J. David, and O. Iismaa, 1962. The determination of chromic oxide in feces samples by atomic absorption spectrophotometry. J. Agr. Sci. 59:381.

Willingam, H. E., L. S. Jensen, and J. McGinnis, 1959. Studies on the role of enzyme supplements and water treatment for improving the nutritional value of barley. Poultry Sci. 38: 539-544.

Wise, A., 1983. Dietary factors determining the biological activities of phytase. Nutr. Abs. and Rev. 53:791-806.

Yi, Z. E. T. Kornegay, and D. M. Denbow, 1996. Effect of microbial phytase on nitrogen and amino acid digestibility and nitrogen retention of turkey poults fed corn-soybean meal diets. Poultry Sci. 75:979-990.

Young, K. M., and P. M. Foegeding, 1993. Acetic, lactic and citric acids and pH inhibition of *Listeria monocytogenes* Scott A and the effect on intracellular pH. J. Appl. Bacteriol. 74:515-520.

Zanella, I., N. K. Sakomura, F. G. Silversides, A. Fqueirdo, and M. Pack, 1999. Effect of enzyme supplementation of broiler diets based on corn and soybeans. Poultry Sci. 78:561-568.

Zyla, K., D. Gogol, J. Koreleski, S. Swiatkiewicz, and D. R. Ledoux, 1999. Simultaneous application of phytase and xylanase to broiler feeds based on wheat: *in vitro* measurements of phosphorus and pentose release from wheats and wheat-based feeds. J. Sci. Food Agr. 79:1832-1840.

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