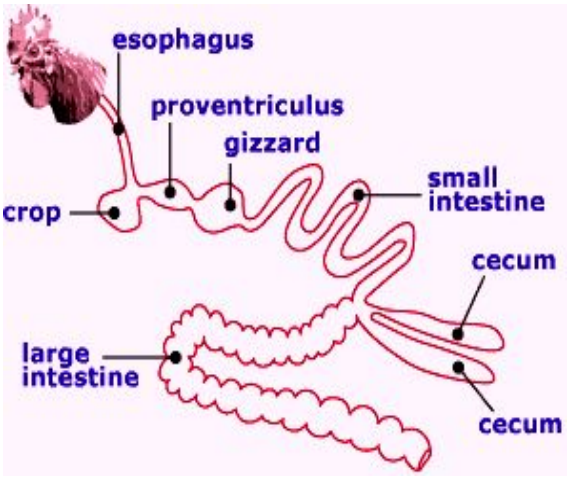
PROTEASY

PROTEASE FOR USE IN POULTRY

INTRODUCTION

Most microorganisms and plants can biosynthesize all 20 standard amino acids, while animals (including humans), must obtain some of the amino acids from the diet. Key enzymes in the animals' body amino acid biosynthetic for specific amino acids are not present at all or not present in enough quantity in animals. The amino acids that an organism cannot synthesize, or synthesized at an insufficient quantity, are referred to as essential amino acids. If amino acids are present in the environment, microorganisms can conserve energy by taking up the amino acids from their surroundings and downregulating their biosynthetic pathways and thus they start requiring outside sources of these amino acids.

In animals, amino acids are obtained through the consumption of foods containing protein. Ingested proteins are broken down through digestion, which typically involves denaturation of the protein through exposure to acid and hydrolysis by enzymes called proteases. Some ingested amino acids are used for protein biosynthesis, while others are converted to glucose through gluconeogenesis, or fed into the citric acid cycle. This use of protein as a fuel is particularly important under starvation conditions since it allows body proteins - particularly those found in muscle - to be used to support life. Amino acids are also important dietary sources of nitrogen.
(http://www.dsm.com/en_US/downloads/dnpna/Proteases_Potential_for_Use_in_Poultry_Nutrition.pdf)



Digestion is the process of breaking down large, complex molecules, as provided by the birds' feed, into smaller components that can be absorbed into the portal blood system. The process involves changes in both physical and chemical structures of most dietary components. Poultry feeds consist of a complex array of particles differing not only in chemical composition, but also in size, hardness, solubility and ionic characteristics.

Under ideal conditions, this array of particles and chemicals with different characteristics degrade slowly in a step-wise manner as feed passes from the mouth to the large intestine. Particle breakdown is a constant process, although the gizzard provides the major site of this activity.

In 1877, Moritz Traube proposed that (i) protein - like materials catalyzed fermentation and other chemical reactions and (ii) they were not destroyed by doing such things. This was the beginning of the recognition of what we call enzymes today.

Enzymes are largely responsible for molecular degradation, although their pH greatly influences their efficacy.
When digestion is reduced, there will be reduced bird growth and/or increased feed intake. Indigestion may also cause problems with manure/litter management, because non-digested residues in the large intestine often adsorb more water or produce feces that are more viscous.

Animal feed contains Cereals and Cakes.
Cell walls of cereals are primarily composed of carbohydrate complexes referred to as Non Starch Polysaccharides (NSP).

ANFs present in these NSP (like ß-glucans and arabinoxylans) are non digestible and form high-molecular-weight viscous aggregates in the gastrointestinal tract.
They

- **Affect the digestive enzymes.**
- **Cause endogenous losses**
- **Reduce the rate of passage.**
- **Stimulates pathogenic microbial proliferation.**

Protease can degrade the proteins.

ANF

PALM

The anti-nutritional factors of the palm kernel cake as discussed by Aletor (1999) are those chemical compounds synthesized inherently or naturally by one organism, acting as stimulators or inhibitors; the factors are phytic acid, tannic acid, oxalate contents and phytin phosphorus.

Anti-nutritional factors of Palm Kernel Cake samples

Anti-nutrients		E1	E2	Mean	SD	CV (%)
Tannic acid (%)	0.35	0.44	0.40	0.064	16.0	
Phytin Phosphorus (mg/g)		6.50	6.73	6.62	0.163	2.46
Phytic acid (mg/g)		23.07	23.90	23.49	0.587	2.50
Oxalate (mg/g)	5.04 5.	22 5.	13	1.273	24.18	

E1 and E2 are duplicate determinations.
SD = Standard Deviation; CV = Coefficient of variation at P < 0.05 significant level.

SOY

Soyabean seeds have certain anti-nutritional factors like trypsin inhibitors, protein inhibitors, protease inhibitors, phytohemagglutinin, saponins, goitrogen and estrogenic factors that can affect the production performance of chickens. But they are all thermo labile and can be destroyed by roasting, heating or autoclaving.

Sesame/Til

Sesame (sesamum indicum) meal contains 40% protein and 8% crude protein fiber. The protein is rich in arginine, leucine and methionine but low in lysine. Since the sesame meal is deficient in lysine its combination with Soyabean meal appears to be useful. Sesame meal has high calcium and phosphorus content but their availability is low because of higher phytate content in the hulls of the seed. The phytic acid reduces the availability of calcium, zinc, magnesium, copper etc. from the diet. Nevertheless, the meal can be included in poultry diets up to 15% in combination with other lysine rich oil cakes.

Sunflower

Sunflower meal is generally not recommended be yond 20% in the diets may be due to high crude fiber content in the meal and also due to the presence of a polyphenolic compound called chlorogenic acid which inhibits the activity hydrolytic enzymes. To overcome this problem, supplementation of additional methionine and choline in the diet is suggested.

Cotton seed

Quality of cotton seed meal is poor due to the presence of gossypol, phenol like compounds that are present in the pigment gland of cotton seed. Gossypol inhibits the activity of digestive enzymes and reduces the palatability of diet. Mechanical pressing of seed followed by solvent extraction reduce the gossypol content to 0.02-0.5% level. Dietary levels of gossypol up to 0.015% levels are believed to be safer in poultry. Detoxification cotton seed meal with solvent mixture containing hexane, acetone and water were found to be useful after initial cooking of the meal. Addition of ferrous Sulphate at the rate of 4 parts to one part of gossypol prevented yolk discoloration of the eggs due to gossypol. Cotton seed has a desirable profile of amino acids except lysine and the digestibility of cotton seed meal is poor due to higher crude fiber.

Caster seed

The protein content of caster (Ricinus communis) seed meal varies from 21 to 48% depending upon the extent of decertification and oil extraction. It has an ideal amino acid profile with moderately high cystine, methionine and isoleucine. But its antinutritional substances called the ricin, ricinine and an allergen restricts its use in poultry even at low levels of inclusion. Processing of caster meal by heating at 80 c temperature in the presence of GN ammonia or an acid or alkali reduces the toxic content substantially. Detoxified meal needs to be effectively corrected for lysine.

G.I Tract Region	Enzyme (or secretion)	Substrate	End Product	pH
Mouth	Saliva	Lubricates and softens food		
Crop	Mucus	Lubricates and softens food		4.5
Stomach (Gizzard and proventiculus)	HCl	Lowers stomach pH		2.5
	Pepsin	Protein	Polypeptides	

Duodenum	**Trypsin, Chymotrypsin and Elastases** **Carboxy-petidases** **Collagenase**	**Proteins, Peptones and Peptides** **Peptides** **Collagen**	**Peptones, Peptides and amino acids** **Peptides and amino acids** **Peptides**	**6 to 6.8**
Jejunum	**Peptidases** **Polynucleotidase**	**Peptides** **Nucleic acids**	**Dipeptides and amino acids** **Mono-nucleotides**	**5.8 to 6.6**

Posttranslational glycosylation has been reported to protect enzymes from deactivation caused by high temperatures and proteinases
(Olsen and Thomsen, 1991).

All enzyme feed additives are considered either food additives or GRAS substances

The activity of enzymes retains more than 95% when stored at the temperature of 25 Deg C upto 3 months

PROTEINS:

Protein and amino acid availability are of greatest concern in animal and vegetable protein ingredients. Protein content and availability from cereals and their by-products seem to be more consistent and little affected by processing conditions.

Feedstuff	C. Protein (%)	Digestibility (%)			
		C. Protein	Lys	Met	Cys
Vegetable sources (cereals)					
Yellow maize	8	82 - 86	81	91	85
Wheat	12	78 - 82	81	87	87
Barley	10	70 -82	78	79	81
Sorghum	10	67 - 72	78	89	83
Vegetable sources (oil seed meals)					
Peanut meals	49	88 - 91	83	88	78
Soybean meals	46	83 - 87	91	92	82
Cottonseed meal	43	61 - 76	67	73	73

Animal sources					
Blood meal	88	82-92	86	91	76
Fish meal	66	86 - 90	88	92	73
Meat meal	60	75 - 80	79	85	58
Feather meal	87	36 - 77	66	76	59

FATS:

G.I Tract Region	Enzyme (or secretion)	Substrate	End Product	pH
Mouth	Saliva	Lubricates and softens food		
Crop	Mucus	Lubricates and softens food		4.5
Stomach (Gizzard and proventiculus)	HCl	Lowers stomach pH		2.5
	Lipase	Fats	Fatty acids, mono-glycerides and glycerol	
Duodenum and Jejunum	Bile	Fats	Emulsification	
	Lipase	Fats	Fatty acids, mono-glycerides and glycerol	5.8
	Cholesterol esterase	Fatty acid - cholesterol esters	Fatty acid, cholesterol	to 6.6

	Digestibility		Metabolizable energy (kcal/kg)	
Fat Type/Age	0-21d	>21d	0-21d	>21d
Tallow	80	86	7400	8000
Poultry Fat	88	97	8200	9000
Fish oil	92	97	8600	9000
Vegetable oil	95	99	8800	9200

Coconut oil	**70**	**84**	**6500**	**7800**
Palm oil	**77**	**86**	**7200**	**8000**
Vegetable soapstock	**84**	**87**	**7800**	**8100**
Restaurant grease	**87**	**96**	**8100**	**8900**

Protease:

A protease (also termed peptidase or proteinase) is any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein.

PROTEASES

Endopeptidases (proteinases)	**Exopeptidases(peptidases)**	
1. Serine proteases eg trypsin, chymotrypsin, subtilisin	**Aminopeptidases**	**Carboxypeptidases**
2.Cysteine proteaseseg papain, cathepsin B	**aminopeptidases I**	**Serine carboxypeptidases**
3.Aspartic proteaseseg pepsin, chymosin	**Aminopeptidases II**	**Cysteine carboxypeptidases**
4.Metalloproteases eg thermolysin, carboxypeptidase A	**Aspartic carboxypeptidases**	

Classification of proteases *(Source : Rao et al., 1998)*

Proteases are currently classified into six broad groups:

1. **Aspartate proteases**
2. **Cysteine proteases**
3. **Glutamic acid proteases**
4. **Metalloproteases**
5. **Serine proteases**
6. **Threonine proteases**

The threonine and glutamic-acid proteases were not described until 1995 and 2004, respectively. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the cysteine and threonine (proteases) or a water molecule (aspartic acid, metallo- and glutamic acid proteases) nucleophilic so that it can attack the peptide carboxyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine, or threonine as a nucleophile.

Within each of the broad groups proteases have been classified, by Rawlings and Barrett, into families of related proteases. For example within the serine proteases families are labelled Sx where S denotes the serine catalytic type and the x denotes the number of the family, for example S1 (chymotrypsins). An up to date classification of proteases into families is found in the MEROPS database

Alternatively, proteases may be classified by the optimal pH in which they are active:

- **Acid proteases**
- **Neutral proteases involved in type 1 hypersensitivity. Here, it is released by mast cells and causes activation of complement and kinins.[3] This group includes the calpains.**
- **Basic proteases (or alkaline proteases)**

Proteases [serine protease (EC. 3.4.21), cysteine (thiol) protease (EC 3.4.22), aspartic proteases (EC 3.4.23) and metallo-protease (EC 3.4.24)] constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market.

Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases and among bacteria, *Bacillus* sp are specific producers of extra-cellular proteases

Alkaline proteases
Strains of *Bacillus*, Steptomyces*, Aspergillus* are the major producers of alkaline proteases.

Neutral proteases
Neutral proteases are produced by bacteria (*Clostridium histolyticum*, *Streptococcus* spp., *Bacillus subtilis*, *B. cereus*, *B. megaterium*, *B. stearothermophilus*, *B. thuringiensis*, *B. pumilus*, *B. polymyxa*, *B. licheniformis*, *B. amyloliquefaciens*. *B. stearothermophilus*, *Pseudomonas aeruginosa*, *Streptomyces griseus*) and fungi (*Aspergillus oryzae*, *A. sojae*, *Penicillium* spp., *Pericularia oryzae*). These microbial neutral proteases are either cysteine or metalloproteases.

Acid Proteases
***Alcaligens*, *Bacillus*, *Corynebacterium*, *Lactobacillus*, *Pseudomonas*, *Serratia*, *Streptococcus*, and *Streptomyces* are the bacteria, and *Aspergillus*, *Candida*, *Coriolus*, *Endothia*, *Endomophthora*, *Irpex*, *Mucor*, *Penicillium*¸*Rhizopus*, *Sclerotium*, and *Torulopsis* are the some of the fungi producing rennin like proteases which find applications in cheese processing.**

Adjuvant for Protease:

Protease activity was stimulated by Mn^{2+} and Ca^{+2}. Several results suggest that these metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active conformation of the enzyme at higher temperatures.
(Beg, Q.K.; Gupta, R. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enz. and Microbial Techn.*, 32: 294-304, 2003.).

Similar effects of Mn^{2+} on the activity of proteases were also observed by Rahman *et al.* , and by Manachini *et al.*

Protease Inhibitors

The activity of proteases is inhibited by protease inhibitors. One example of protease inhibitors is the serpin super family, which includes alpha 1-antitrypsin, C1-inhibitor, antithrombin, alpha 1-antichymotrypsin, plasminogen activator inhibitor-1, and neuroserpin.

Natural protease inhibitors include the family of lipocalin proteins, which play a role in cell regulation and differentiation. Lipophilic ligands, attached to lipocalin proteins, have been found to possess tumor protease inhibiting properties. The natural protease inhibitors are not to be confused with the protease inhibitors used in antiretroviral therapy. Some viruses, with HIV/AIDS among them, depend on proteases in their reproductive cycle. Thus, protease inhibitors are developed as antiviral means.

Degradation of Protease

Proteases, being themselves proteins, are known to be cleaved by other protease molecules, sometimes of the same variety. This may be an important method of regulation of protease activity.

Protease research

The field of protease research is enormous. Barrett and Rawlings estimated that approximately 8001 papers related to this field are published each year.

Protease Bonds with alpha 2-macroglobulin to support immune function when taken on an empty stomach.

Protease is responsible for digesting proteins in food, which is probably one of the most difficult substances to metabolize. Because of this, protease is considered to be one of the most important enzymes that we have. If

the digestive process is incomplete, undigested protein can wind up in the circulatory system, as well as in other parts of the body.

When protease is present in higher quantities, it can help to clean up the body by removing the unwanted protein from the circulatory system. This will help to clean up the blood stream, and restore the energy and balance.

One of the tricks of an invading organism is to wrap itself in a large protein shell that the body would view as being "normal". Large amounts of protease can help remove this protein shell, and allow the body`s defense mechanisms to go into action. With the protective barrier down, the immune system can step in and destroy the invading organism.

Protease refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. Proteases differ in their ability to hydrolyze various peptide bonds. Each type of protease has a specific kind of peptide bonds it breaks. Examples of proteases include: fungal protease, pepsin, trypsin, chymotrypsin, papain, bromelain, and subtilisin.

Proteolytic enzymes are very important in digestion as they breakdown the protein foods to liberate the amino acids needed by the body. Additionally, proteolytic enzymes have been used for a long time in various forms of therapy. Their use in medicine is gaining more and more attention as several clinical studies are indicating their benefits in oncology, inflammatory conditions and immune regulation.

Contrary to old beliefs, several studies have shown that orally ingested enzymes can bypass the conditions of the GI tract and be absorbed into the blood stream while still maintaining their enzymatic activity.

Commercially, proteases are produced in highly controlled aseptic conditions for food supplementation and systemic enzyme therapy. The organisms most often used are Aspergillus niger and oryzae.
Measured in HUT (Hemoglobin Units in a Tyrosine Base).

Gastrointestinal section	pH	Residence time/minutes
Crop	4,5-5,3	45
Proventriculus, gizzard	2,0-4,5	70
Ileum	5,6-7,9	160-200
Caecum	5,8-6,8	120
Colon, rectum	6,3-7,7	30-50

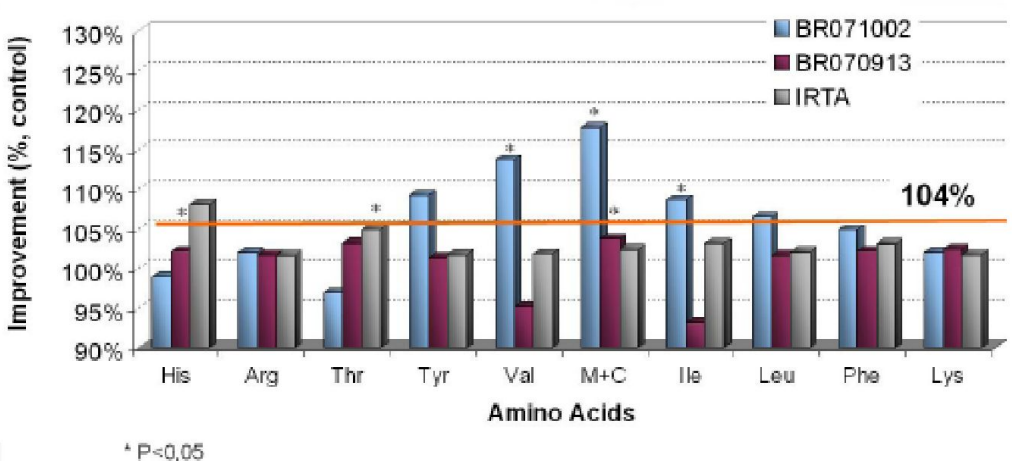
pH and residence time of feed in gastrointestinal tract

PROTEASES USED IN POULTRY

Following Data is available relating ProAct of DSM.

The protease (ProAct®, DSM) is an alkaline serine protease derived from *Nocardiopsis prasina* and the production strain *Bacillus licheniformis*. Pepsin stability experiments find that 97% of this protease remains intact and active after being exposed to pepsin for 1.5 hrs, pH 3 and at 40oC (Novozymes Internal Reports).
The protease was found to be effective across a wide range of peptide bonds and considered to be a non-specific protease (Fischer et al., 2009. This protease is characterized by an optimal pH range that begins at pH of 5-6, and continues into the area of alkalinity. This range in operating pH complements existing endogenous proteases such as pepsin and others that operate optimally in an acidic pH. Carvalho et al. (2009 a and b) and Bertechini et al. (2009 a and b) presented ileal digestibility results with this protease.
This work confirms the ability of this protease to improve ingredient protein digestibility.

These researchers used the NFE diet or basal diet substitution methods to determine amino acid digestibility in different ingredients. This method is patterned after the procedure developed by Matterson et al. (1965) in which birds were fed a reference diet with significant portions being replaced by test ingredients. Across all ingredients tested, corn, soybean meal, full fat soybean meal and meat and bone meal, the protease improved amino acid digestibility. The improvement over the non-protease control ranged from about 2% to 14%.



Apparent essential amino acid digestibility improvement in different diet with 200 ppm ProAct® protease. Adapted from Favero et al., 2009, Maiorka et al., 2009 and Vila and Broz, 2008.

The digestibility improvement across essential amino acid was approximately 4% with the use of 200 ppm ProAct® protease.

In a study done at the University of Maryland in 2009 (Angel, 2010) with Ross 708 straight run broilers were fed corn soy diets with graded concentrations of this protease from 7 to 22 d of age in batteries. Six diets were fed. A positive control (PC) that was formulated based on AgriStats (2008) met or exceeded all NRC (1994) nutrient recommendations. The PC diet contained 22.5% crude protein. All other diets were negative control diets with the protease (0, 100, 200, 400, 800 ppm). The negative control diet contained 20.5% crude protein and a 9% reduction in the concentration of Lys, Met, TSAA and Thr was forced into the diet. All diets were isocaloric. The content of the distal ileum was removed with water, freeze dried, and ground and analyzed for marker, nitrogen and amino acids. Analysis of the diets confirmed levels close to formulated levels. There was no clear dose response for any of the essential amino acids. Overall, the improvement in essential amino acid digestibility due to the protease was 4.8% when 200 ppm was used with the greatest impact seen in threonine. The non essential amino acids were improved by an average of 3.72% with Asp and Ser being the most positively affected Across several studies, threonine digestibility is improved to the greatest extent with the ProAct® protease. This is true for most individual ingredients, as well as corn/SBM diets fed for ileal amino acid digestibility determinations.

DATA ON PROTIGEST

1. Source of origin (wheather Fungal or Bacterial)

Fungal and Bacterial

2. Type of Fermentation used for harvesting

Solid state fermentation

3. Nature of the product (Acid or Alkaline)

Mix of Acid, Neutral and Alkali; Blend of Endopeptidases (proteinases) and Exopeptidases(peptidases)(C-Terminal and N-Terminal)

4. Which nature Protease is suitable for mixing in poultry feeds.

Proteases perform a variety of roles in biology. These enzymes function in important physiological processes, including homeostasis, apoptosis, signal transduction, reproduction and immunity (Hedstrom, 2002). In addition, proteases are involved in blood coagulation and wound healing. To accomplish a wide range of tasks, over 500 proteases exist in humans; a similar number is present in other organisms.

From a nutritionist's perspective, the hydrolysis of proteins to individual amino acids and peptides in the intestinal tract is a key function for proteases. Several intestinal proteases exist, and comprise a "protease system" in the intestinal tract for the utilization of various protein sources.

Hence a suitable mix of several Proteases is required.

5. If it is a combination, what is the RATIO of acid type to alkaline type.

57.5% Acidic Protease(pH 4.0), 37.5% Neutral Protease (pH 6.0) and 5% Alkaline (pH 8.0) Protease combination is found to be the best option .

6. Degree/ Extent of IN-VITRO hydrolysis of feed proteins and the probable time- period taken.

Depends on the source of protein, will hydrolyze up to 80% of soya protein

Passage of hydrochloric acid, produced in the proventriculus, and peptides through the proventriculus and gizzard into the duodenum stimulates the release of the hormones secretin and pancreozymin from the mucosa of the duodenum (Rothman and Wells, 1967). These hormones promote the secretion of pancreatic juice containing a number of enzymes and bicarbonate ions. The production of an alkaline solution quickly neutralizes the acid entering the duodenum (Barash et al., 1993). Small intestinal enzymes function best at pHs close to neutral or slight below neutral and thus, insufficient alkaline bile, lowers enzyme activity in the intestine (Zentler-Munro, 1985).

Pancreatic proteases are secreted from the pancreas only in the form of zymogens. All known cellular proteases are synthesized as zymogens, or the inactive precursor, toprevent unwanted protein degradation at the point of origin or thereabouts (Kahn andJames, 1998). The conversion of the zymogens to the active protease requires low pH(autocatalysis) or limited proteolysis. Primary pancreatic zymogens are trypsinogen, chymotrypsinogens A and B, proelastase, and procarboxypeptidases A and B. Trypsin is activated after being attacked by enterokinase, found in the brush border membrane. The active trypsin then hydrolyses bonds in the other zymogens, releasing the active enzymes.

From a practical nutritionist's standpoint, the value of ingredients as a protein (amino acid) source generally comes down to the total amino acids it contains, the ratio of amino acids and availability of the amino acids (aside from cost, ingredient accessibility, etc.).

Numerous studies and tables on protein and amino acid availability (the digestibility, absorption and utilization by the animal) have been published. The common thread that runs through such summaries is that ingredient improvements can yet be made in digestibility of protein.

In one large scale study (Ravindran et al., 2005), apparent ileal digestibility was determined for various ingredients for broilers. For cereals, the overall amino acid digestibility coefficient for eight samples of corn was 0.81 (range 0.77 to 0.85). For soybean meal, the digestibility coefficient was 0.82 (range 0.81 to 0.83). In

particular, the groups of meat and bone meal (avg 0.62) and meat meal samples (avg digestibility coefficient 0.65) showed low amino acid digestibility values. This was accompanied by marked variation.

Similarly, University of Illinois work has reported a number of studies on amino acid digestibility of ingredients. Low values and high variation for meat and bone meals is typical
(Parson et al., 1997; Parsons et al., 1998).

More recent collaborative work with The Ohio State University, Purdue University and University of Illinois further verifies such data
(Adedokun et al., 2007).

(http://www.dsm.com/en_US/downloads/dnpna/Proteases_Potential_for_Use_in_Poultry_Nutrition.pdf)

7. Stability during storage and against PELLETIZATION.

Normal Enzymes: Storage stability 1 year at about 18 Degrees, tolerant to pellet temperature of 80oC for 1 min. and cooling time of 5 min
PROTIGEST: Since Protigest contains a protectant, Colloidal Ca^{++} : Storage stability 1 year at about 18 Degrees, tolerant to pellet temperature of 95°C for 1 min. and cooling time of 5 min

8. Maximum CONCENTRATION/ACTIVITY per GRAM

1,75,000 HUT/g

9. ANY OTHER SPECIFICATIONS?

Light brown colored Liquid

10. HOW IT DIFFERS WITH OTHER ENZYMES IN THE MARKET?

Thermo-stable and broad spectrum PROTIGEST aggressively works in the entire GI tract irrespective of pH variations.
PROTIGEST complements poultry's endogenous enzymes.

Handling and Safety

Enzymes are proteins, and as is the case with all proteins, people can have allergic reactions when exposed to them. Most enzymes are in a dry form, so airborne contamination and exposure is possible. It is important that workers be aware of proper handling techniques.
However, if exposure is a concern based upon the specific manufacturing environment, preparations are available that are either in a liquid form, or specially processed to minimize the dust

SUGGESTED LEVEL AND METHOD OF USAGE:

50-100 g or ml per MT feed regularly

Ref:

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