CHAPTER 2

LITERATURE REVIEWS

2.1 Pig production in Thailand

Throughout the 1960s and 1970s Thai pig production was dominated by the backyard raising of pig cross-breeds for consumption and the generation of supplementary income. Most pigs in Thailand were traditionally raised by Thai rice farmers to consume farm by-products and wastes and generate extra farm income. Along with buffalo, cattle and poultry, pig production was an important component in an integrated small farm cropping system where buffalo and cattle were used for draught purposes and pigs and poultry for consumption (Murphy and Tisdell, 1995).

The development of pig production from predominantly village-based to a growing commercial industry can be attributed to significant socio-economic change that is associated with an increase in demand for meat such as pork, both domestically and internationally. Pig farming has rapidly increased, especially in eastern and central regions of the country. Since Bangkok is the major market for pork in Thailand, swine production is concentrated in the central provinces around Bangkok, an area which accounts for approximately 36-40% of total pig production. The northern region ranks second, accounting for 26-30% of total national production.

Over the last decade, the importance of commercial pig farms has increased significantly. It is noted that modern operations have at least 400-500 sows, with Landrace and Large White as the most common breeds and Durocs as the preferred sire. It has recently been estimated that about 80% of all pig production is now carried out by commercial enterprises. According to the statistics of Department of Livestock Development, slightly more than 6 million heads of pig in 1989, their numbers in Thailand increased to 8 million in 1991 and reached 10 million in 1997 (Figure 2.1). Partially due to the price instability and disease epidemic, the growth of the industry have been declined during 1998-1999. However, slightly higher production was observed in 2000-2001.

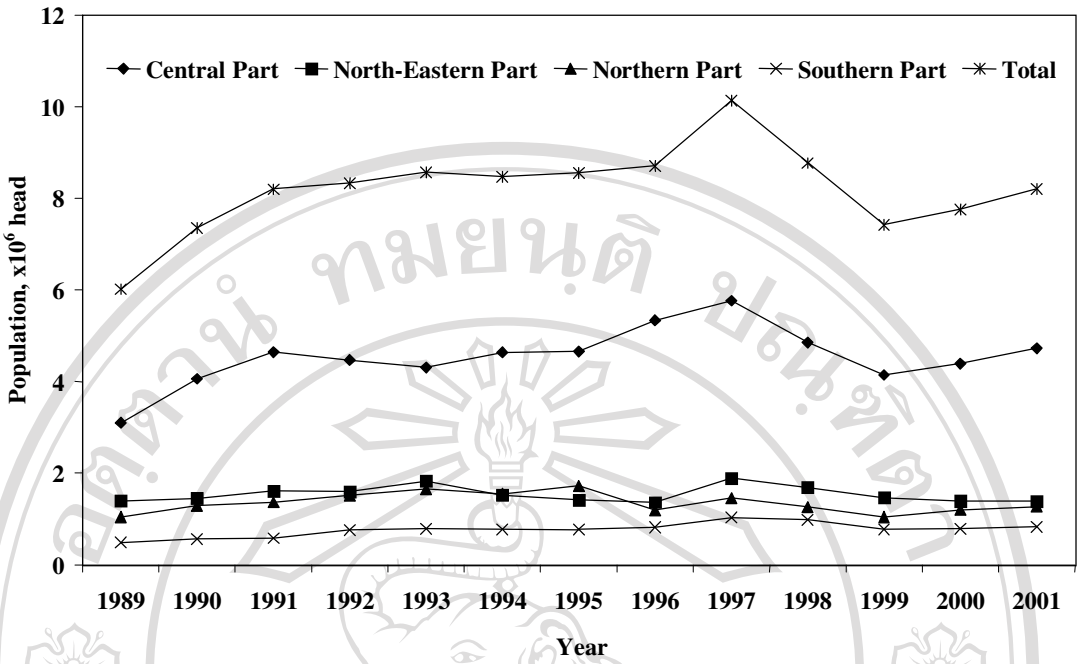


Figure 2.1 Swine production in Thailand (1989-2001) (Provincial Livestock Office, 2003)

2.2 Common feedstuffs

The cereal grains that constitute the bulk of animal feedstuffs also provide 30-60% of dietary amino acids. However, this protein is inadequate, not only in sufficiency but also in amino acid balance. To obtain optimum animal performance, whether it be growth, reproduction or lactation, protein must be added to the diets to provide both as adequate amount of total protein and a balance of amino acid that approaches the ideal amino acid pattern for the species and development stage of animal. The majority of protein ingredients incorporated into animal feeds are supplied by vegetable proteins meals (VPMs), with the oilseed and legumes being the main providers. The vegetable sources of protein vary both in crude protein concentration and in the amino acid composition of the protein.

Use of vegetable protein in animal feed is becoming increasingly important with the restrictions on animal and fish protein sources, this figure is likely to increase in the future. Soybean meal (both as the extracted oil meal and the unextracted 'full-fat' product) represents more than half of the total production of all protein meals. Variability in the nutritional value of soya products is a problem. Furthermore, despite a wealth of information on anti-nutritional factors and their destruction, there is still a

general lack of knowledge relating to how anti-nutritional factors and their levels relate to animal performance in practical feeding situations. Table 2.1 shows the distribution and physiological effects of the various ANFs found in VMPs. The toxicity and effects of the difference ANFs may vary considerably between VMPs and these toxic defects may also vary in potency between animal species. ANFs appear to play a role in the protection of plants against predation of from molds, bacteria, insects and birds by disturbing the digestive processes of these organism, and presumably act in a similar manner in domestic animals (Birk, 1989).

2.2.1 Anti-nutritional components of soy products

A number of anti-nutritional and/or allergenic compounds exist in soybeans, such as, trypsin inhibitors, glycinin, β-conglycinin, oligosaccharides, lectins, and saponins (Liener, 1994). These factors have been documented to cause gastrointesinal disturbances, intestinal damage, increased disease susceptibility, and reduced performance. Examples of some of the anti-nutritional component differences between soy products is shown in Table 2.2.

2.2.1.1 Protease inhibitors

Protease inhibitors are protein molecules, which have the ability to inhibit the activity of protease enzymes within the alimentary tract. In raw soybeans these account for about 6% of the protein content and their main constituents are the heat labile Kunitz inhibitors and the heat stable Bowman-Birk inhibitor. The former is a larger protein molecule (molecular weight 20,000-25,000), which inhibits mainly trypsin. The latter is a smaller molecule (molecular weight 6,000-10,000), which inhibits both trypsin and chymotrypsin (Liener, 1986). The protease inhibitors act through binding on the digestive enzymes making them unavailable for protein hydrolysis. This reduces digestibility of protein and therefore its availability for growth. Digestive proteases in fish have been generally found to be more sensitive to inhibition than those of mammals (Krogdahl *et al*., 1994; Krogdahl and Holm, 1983). Binding of proteases by inhibitors also results in increased pancreatic secretion of these enzymes (Figure 2.2). Pancreatic enzymes contain a high level of sulfur amino

Table 2.1 Distribution and physiological effects on ANFs found in VPMs (adapted from Huisman and Tolman, 1992; Liener, 1994)

Anti-nutritional factor	Distribution	Physiological effect
Protein		
Protease inhibitor	Most legumes	Reduction of trypsin and chymotrypsin activity, impared growth, pancreatic hypertrophy, pancres carcinogen
Lectins	Most legumes	Gut wall damage, immune response, increased endogenous nitrogen loss
Amylase inhibitors	Kidney beans	Impaired digestion of starch
'Anti-genic protein'	Soybean, Kidney beans	Immune response, interference with gut wall integrity
Polyphenols		
Tannin	Most legumes	Interference with protein and carbohydrate digestibility by formation of protein-carbohydrate complexs
Glycosides		
Vicin / Convicin	Faba beans	Haemolytic anaemia, adverce effect on egg production
Saponins	Soybean	Haemolysis, effect on intestinal permeability
Glucosides		
Glucosinolates	Rapeseed	Impaired iodine utilization (goitorenicity), reduced palatability and growth
Cyanogens	Linseed, traces in kidney bean and peas	Respiratory failure
Alkaloids		
Quinolizidine	Lupin	Neural disturbances, depressed growth , reduced palatability
Others ANFs		
Phytate	Most legumes	Formation of complexes with minerals and protein, depresses mineral absorption
Gossypol	Cottonseed	Anaemia due to formation of iron complexes, reduced egg weight
Saponins	Rapeseed	'Fish' taint in eggs
Oligosaccharides (NSP)	Soybean, peas, faba beans, *Phaselolus* beans	Flatulence, diarrhea, diarrhea, discomfort

acids, mainly cystine. This creates an additional requirement for sulfur amino acids. The most common method for protease inhibitor destruction is heat treatment, which results in their denaturation. The degree of denaturation depends on the extent and severity of the treatment. Toasting (steam cooking of soybean meals) reduces protease inhibitor activity (Anderson and Wolf, 1995). As such, apparent digestibilities of dry matter and nitrogen were reported to increase in pig fed raw soybean meal when the level of soybean trypsin inhibitors (SBTI) was decreased by thermal processing (Qin *et al*., 1996).

Table 2.2 Some anti-nutritional components of soy products (Russett, 1998)

	Raw soybeans	**Toasted soybean meal**	**Toasted soy flour**	**Traditional soy protein concentrate[a]**	**Extruded soy protein concentrate[a]**
Trypsin inhibitor, mg TI/g	45-50	5.0-8.0	5.0-8.0	<0.4	<1.25
Glycinin antigen[b]	>15 184,000	13-15 66,000	13-15 66,000	<2 <30	<1 <5
β-conglycinin antigen[b]	>15 69,000	13-15 16,000 +/-	13-15 16,000 +/-	<1 <10	<1 <1
Lectins, mcg/g	3,600	10-200	10-200	<0.1	<0.1

[a] Profine, Profine E and Profine II, respectively.

[b] Hemagglutination Assay (wells of inhibition) and ELISA (ppm), first and second line, respectively.

[c] Varies within and between countries, dependent on processing.

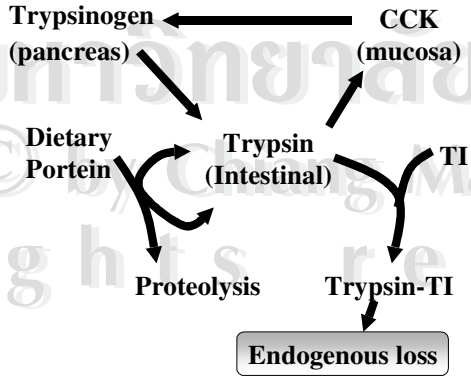


Figure 2.2 Mode of action of trypsin inhibitor (TI).
CCK=cholecystokinin (Liener, 1989)

2.2.1.2 Lectin

Lectin is a component of soybeans that is believed to be an anti-nutritional factor (Schulze *et al*., 1995). Lectins are glycoproteins that have the ability to bind to cellular surfaces via specific oligosaccharides or glycopeptides (Oliveira *et al*., 1989) and have a relatively high binding affinity to small intestinal epithelium (Pusztai, 1991). Furthermore, lectins can produce structural changes in the intestinal epithelium and resist gut proteolysis (Pusztai *et al*., 1990). These changes can result in impairment of brush border continuity and ulceration of villi (Oliveira *et al*., 1989), which may result in increased endogenous nitrogen losses (Schulze *et al*., 1995; Oliveira; Sgarbierri, 1986) and depressed growth rate in young animals (Pusztai *et al*., 1990). Thus, the growth depressant effect of lectins is believed to be due primarily to their damaging impact on intestinal enterocytes (Lorenzsonn and Olsen, 1982; Pusztai, *et al*., 1979) and through appetite depression (Liener, 1986). Research has shown that the detrimental effects of lectins may be lessened with proper heat processing of conventional soybeans (Higuchi *et al*., 1984). The lectins in soybeans are tetrameric glycoproteins that have a specific affinity to terminal N-acetyl-D-glucosamine and D-galactose (Schulze *et al*., 1995). These lectins were originally referred to as hemagglutinating factor or soyin, and it was estimated that they accounted for one-half of the growth inhibition produced by raw soybeans fed to rats (Liener, 1953).

2.2.1.3 Soy antigens

Soy protein specifically glycinin and conglycinin have been associated with changes in piglet performance of animal feed with soy protein concentrate and soybean meal. About 70% of the protein in soybean seed is present as storage proteins (SP) glycinin and â-conglycinin, which are also called 11S and 7S proteins, respectively. Glycinin accounts for about 60% of SP and â-conglycinin for the remaining 40%. Glycinin is composed of five subunits whose concentration of S-amino acids ranges from 3 to 4.5%. In contrast, â-conglycinin, which is composed of three subunits (á' [76 kDa], á [72 kDa], â [53 kDa]), contains less than 1% of S-containing amino acids. The mature â-subunit of â-conglycinin contains only one cysteine residue and no methionine among about 470 amino acids (Hettiarachchy and

Kalapathy 1998; Sexton *et al*. 1998; Shimoyama *et al*. 1998; Marsman *et al*. 1997). Henning (1981) have reported the importance of postnatal gut development for the digestion and absorption of nutrients to support growth and as a defense against infection. Exogenous variables, such as diet, microbial colonization, stress, and raising environment all effect postnatal gut development. The negative influence of dietary soybean meal on the intestinal tract lining of pigs was also reported by Dunsford *et al*. (1989). They showed that feeding high concentrations of soybean meal to the pig postweaning had a detrimental effect on the small intestine mucosa and on blood serum anti-soy antibody titer. The influence of dietary soy protein concentrate versus soybean meal on piglet performance has been reviewed by Russett (1997a,b). These changes have been associated with soy antigens, specifically glycinin and beta-conglycinin. Li *et al.* (1991a) showed pigs fed SBM had shorter villus height than pigs fed either milk protein or the other soybean products (Table 2.3). It also found that pig fed SBM had higher Ig G titers than pigs fed milk protein. It was suggested that glycinin and β-conglycinin was absorbed form the digesta to the gut lumen and stimulated the systemic immune system.

Table 2.3 Effect of feeding different soybean products on gut morphology and serum antibody immunoglobulin G titers to soy protein of pigs (Li *et al*., 1991a).

Criteria	**Milk protein**	**Soybean meal**	**Soy protein concentrate**	**Extrude soy protein concentrate**	**Soy protein isolate**
Villus height (μm)	266.2[a]	175.0[b]	207.3[a]	230.0[a]	216.8[a]
Perimeter length (μm)	731.4[a]	518.0[b]	667.3[a]	666.3[a]	609.7[a]
Titer	3.86[b]	6.67[a]	3.83[b]	4.25[b]	2.56[c]

[a, b, c] Means within column with different superscripts are significantly different (P<0.05).

Further, soy protein mediated changes in gastrointestinal tract morphology have been reported in rats (Govers *et al*., 1993). The influence of soy antigens on intestinal tract villi can be visualized in Figure 2.3. With damage such as this to the digestive tract lining reducing the absorptive surface and disease barrier integrity, the

negative effect of soy antigens on all young animals' performance can be understood. For example, Draéu *et al*. (1999) characterized the influence of soy on early weaned piglets stating that hypersensitivity to soybean proteins is associated with damage to the small intestinal lining that may be due to a local cellular immune response. It was suggested that these observations could indicate that soybean meal proteins could alter the balance of competition between gut bacteria and allow more pathogenic strains to establish in the intestine.The interaction of dietary components and gut bacteria with gut epithelium may be facilitated by changes in intestinal coating which occur at weaning. Mucins protect the lining of the small intestine of young animals from invasions by pathogens like *Escherichia coli* and *Salmonella*. Mathew *et al*. (1997) found significant changes in intestinal mucins following weaning, that would result in lower resistance to intestinal pathogens.

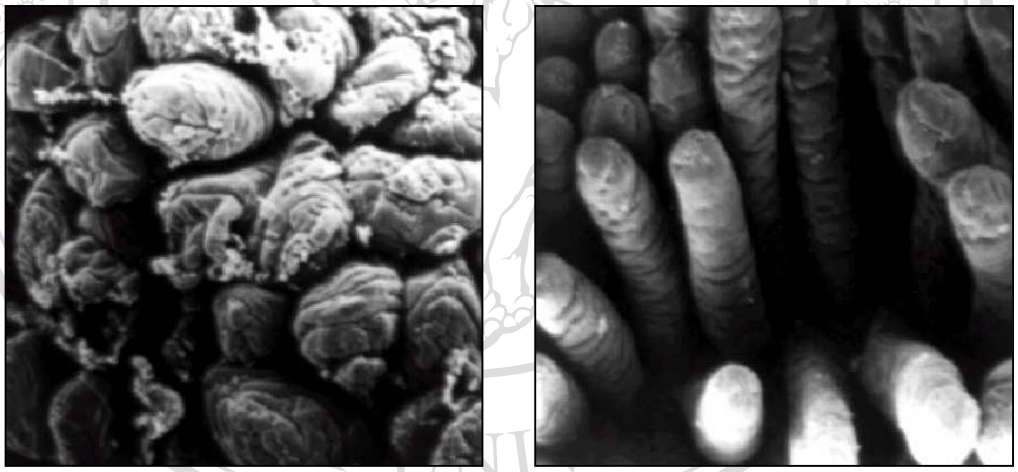


Figure 2.3 Scanning electron micrographs of mid-jejunal sections of the small intestine of two soy-sensitive animals challenged with either a soy flour (left) or an ethanol extracted traditional soy concentrate (right) containing diet (Russett, 1998).

2.3 The effect of nutrient impacts on water quality

Livestock manure can provide valuable organic material and nutrients for crop and pasture growth. However, nutrients contained in animal manure can degrade environmental quality if they enter the water. There is growing concern about the large amounts of manure nutrients being generated by large animal feeding operations and the potential for some of the nutrients to enter water resources and impair water quality. Pollution Control Department (PCD) of Ministry of Natural Resources and

Environment, Thailand controlled the effect of livestock farm especially in the commercial pig farm on the water quality standard by set the effluent standards. The standard effluent for pig farm is summarized in Table 2.4, total Kjedahl nitrogen (TKN) was one of the parameters that high important but there are not concerned with phosphorus. In developed country, USA, nitrogen is primarily a problem in brackish or salt water, while phosphorus is primarily a problem in fresh water. EPA reports that nutrient pollution is the leading cause of water quality impairment in lakes and estuaries, and is the second leading cause in rivers, behind sediment. The National Water-Quality Assessment Program found that the highest concentrations of nitrogen and phosphorus in streams occurred in basins dominated by agricultural uses. High concentrations of nitrogen and phosphorus in these streams were correlated with inputs from fertilizers and manure used for crops and from livestock wastes. The Clean Water Act (CWA) and the Coastal Zone Management Act (CZMA) are the two primary federal laws that deal with animal waste regulation. Under the CWA, the concentrated animal feeding operations (CAFOs) may be treated as point sources of pollution and regulated by a permit system (Table 2.5).

Table 2.4 Effluent standard for pig farm (Pollution Control Department, no date).

		Maximum standard values for	
Parameters	**Unit**	**large farm**	**Small and medium farm**
pH	-	5.5-9.0	5.5-9.0
Biochemical Oxygen Demand (BOD)	mg/l	60	100
Chemical Oxygen Demand (COD)	mg/l	300	400
Suspended solids (SS)	mg/l	150	200
Total Kjedahl Nitrogen (TKN)	mg/l	120	200

Large farm is more than 600 Livestock Unit (LU.), Medium farm is 60-600 LU., Small farm is 6-<60 LU., 1 LU. = 500 kg. Weight of breeding pig = 170 kg./head Weight of fattened pig = 60 kg./head Weight of nursling pig = 12 kg./head

Table 2.5 Categories of CAFOs and numbers of animals required to obtain a permit under the CWA in EPA region 6 (U.S. Environmental Protection Agency, 1993).

Categories	If CAFO discharges into other than navigable waters		navigable waters	
	Head	Animal Units[a]	Head	Animal Units[a]
Slaughter or feeder cattle	1,000	1,000	300	300
Mature dairy cattle	700	980	200	300
Swine weighing more than 55 pounds	2,500	1,000	750	300
Horses, stabled	500	1,000	150	300
Sheep or lambs	10,000	1,000	3,000	300
Turkeys	55,000	1,000	16,000	300
Laying hens or broilers with unlimited Continuous flow watering systems	100,00 0	1,000	30,000	300
Laying hens or broilers with liquid manure handling systems	30,000	1,000	9,000	300
Ducks	5,000		1,500	

[a]A unit of measurement for any animal feeding operation calculated by multiplying slaughter and feeder cattle by 1.0, mature dairy cattle by 1.4, swine over 55 pounds by 0.4, sheep by 0.1, horses by 2.0, turkeys by 0.018, and chickens by 0.01 unless a liquid system is used then multiply by 0.033.

2.4 Improving the nutritional value of grain legumes

2.4.1 Plant breeding

Recent advances in plant breeding and genetic engineering have resulted in many new soybean varieties. Some of these new varieties are bred such that anti-nutritional factors are minimized, such as trypsin inhibitors (Han *et al*., 1991) or lectins (Douglas *et al*., 1999). Improvement of the nutrient profile of the soybean has also been accomplished with the development of a high-lysine soybean variety (Parsons and Zhang, 1997). Reduction of condensed tannin content through genetic manipulation of faba bean, for example, has resulted in increased digestibility of dry matter and nitrogen in pigs (van der Poel *et al*., 1992) and a 2.5% increase in feed conversion in broiler chicks (Helsper *et al*., 1996). Plant breeding is, however, a long-term process and the results, at least for the removal of trypsin inhibitors in peas (*Pisum sativum*), have not been consistent. Some cultivars had higher trypsin inhibitor activity than of their parents,

and cultivars derived from the same cross showed different trypsin inhibitor activities, suggesting that the hereditary transmission was not systematic (Leterme *et al*., 1992).

2.4.2 Physical treatments

The physical treatments was widely use for reduction of the negative effects by ANF. They can be grouped into mechanical treatments and heat treatments. Weight gain, feed-to-gain ratio, apparent protein digestibility (APD) and apparent metabolizable energy (AME) were improved by 5.7, 7.0, 16.4 and 13.9%, respectively when growing chickens were fed dehulled-high-tannin peas compared with those fed an untreated pea diet (Brenes *et al*., 1993a). This improvement may occur not only because of the reduction of tannin content but also because of the reduction of fibre content associated with removal of the testa. On the other hand, lectins and trypsin are more concentrated in the cotyledon than in the hull (Valdeouze *et al*., 1980; Marquardt *et al*., 1975), so that dehulling is not an effective method for reducing lectins and proteinase inhibitors.

2.4.3 Heat treatments

Heat causes denaturation of proteinaceous inhibitors. It is a good method of decreasing the activity of lectins, and also that of trypsin and chymotrypsin inhibitors. Several different heat treatments can be applied to reduce the lectin activity (LA) and ` heat-labile'trypsin inhibitor activity (TIA) which generally result in improving the nutritional value of grain legumes (Table 2.6). Brenes *et al*. (1993b) reported that inclusion of 47.5% autoclaved (121°C for 20 min) high-tannin peas (*Pisum sativum*) into a corn-soy basal diet increased the apparent metabolizable energy (AME) and apparent protein digestibility (APD) by 21 and 11%, respectively compared with inclusion of the same amount of unprocessed peas. Application of the same technique to lupin (*Lupinus albus*) increased weight gain by 11% and reduced feed-to-gain ratio by 10% but failed to reach a significant level (Brenes *et al*., 1993a). The results of these two studies suggest that the effect of autoclaving is specific to each legume, and may depend on the concentration of the heat-labile ANF. The biological value of diets containing autoclaved, infrared treated and boiled winged bean was not significantly different. Application of infrared radiation to peas fed to young chickens also resulted

in a significant increased in apparent metabolizable energy (AME), apparent protein digestibility (APD) and starch digestibility (Igbasan and Guenter, 1996).

These treatments are potential methods for improving the nutritional value of grain legumes. The choice of the treatment will, therefore, depend on the availability of facilities and the economic considerations. The effect of heat depends on the temperature and duration of processing (Qin *et al*., 1996; Wu *et al*. 1996a,b; Ichihara *et al*., 1994; van der Poel *et al*., 1990). Excessive heat treatment can result in the reduction of protein solubility and may destroy certain amino acids (van Barneveld *et al*., 1993; Dhurandhar and Chang, 1990; Metebe, 1989; Almas and Bender, 1980). It is therefore importedant to find the exact conditions of heating which maximise the destruction of the ANF and minimize damage to the feed protein. Heat treatments are the most widely used techniques for inactivating ANF, however, as well as potentially destroying nutrients, this method increases the operating cost for a feed company.

Table 2.6 Effect of heat treatments on inactivation of ANF in *Phaseolus vulgaris* beans.

Process	Heating conditions		Reduction ANF		
	Temp (°C)	Time (min)	In trypsin inhibitor activity (%)	In lectin activity (%)	Reference
Autoclaving	121	5	82	100	Kakade and Evans, 1965
	121	30	88	100	Kakade and Evans, 1965
	121	15	100	100	Myer and Froseth, 1983
	121	10	72	ND	Tan *et al*., 1984
Extrusion	145	16	78	98	Myer and Froseth, 1983
Heating, presoaked	88	60	95	99	Dhurander and Chang, 1990
	100	10	95	100	Dhurander and Chang, 1990
	100	10	95	100	Dhurander and Chang, 1990
	100	15	ND	100	Thomson *et al*., 1983
	80	120	ND	100	Thomson *et al*., 1983
Steam heating	105	20	94	95	van der Poel *et al*., 1990
	150	40	97	98	van der Poel *et al*., 1990
	100	15	65	100	Rodriguez and Bayley, 1987
	100	75	97	98	Rodriguez and Bayley, 1987
Toasting	ND	40	94	94	Huisman and van der Poel, 1989

ND = Not determined

2.4.4 Chemical treatments or exogenous enzyme treatments

Enzymes are protein catalysts for chemical reactions in biological systems. Made up of long chains of amino acids with a highly complex molecular structure, they play a key role in the digestive process. Although enzymes are produced by the animal itself and/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. Enzymes used as feed additives are produced through large-scale fermentation processes. More recently some chemical treatments (such as the addition of exogenous enzymes) have been developed to destroy ANF without affecting the composition of the dietary nutrients. The inactivation of trypsin inhibitors, chymotrypsin inhibitors and lectins of raw soybean by incubation with microbial proteases (Huo *et al*., 1993) and extensive hydrolysis of raffinose-family oligosaccharides of ground cow peas by microbial β-galactosidase (Somiari and Balogh, 1993) have been demontrated. This finding open up the possibility to employ enzymes to enhance the quality of grain legumes. Although most evidence concerning enzymatic inactivation of ANF comes mainly from *in vitro* research, they are a good indication of *in vivo* digestion. Enzyme supplement is likely to be a safer, more effective and cheaper method of reducing the negative effects of anti-nutritional factors in grain legumes. The best enzymes to use and the extent of their effectiveness in improving animal performance need to be elucidated.

Caine *et al*. (1998) described a series of *in vitro* studies with a subtilisin protease which showed the potential of the enzyme to solubilized protein in toasted soybean meal and, at the same time, reduce levels of residual soybean trypsin inhibitors (Table 2.7). Similar effects were described for a range of proteases, with differing pH characteristics, examined by Beal *et al*. (1998a).

Table 2.7 Effect of protease addition on total soluble matter, soluble crude protein and soybean trypsin inhibitor level (SBTI) (Caine *et al.*, 1998).

	Control (no enzyme addition)	**+ Protease**
Soluble matter (g/kg)	212.40[a]	356.00[b]
Soluble crude protein (g/kg)	90.50[a]	318.70[b]
SBTI (mg/kg)	3.55[a]	3.08[b]

Mean values with different superscripts (a,b) in the same column indicate significant differences (P<0.05).

The in vitro studies with subtilisin protease under the optimum temperature (50°C), concentration 1 mg/g soybean meal and pH conditions (pH 4.5).

In growth trials, it certainly appears that some of the benefits seen *in vitro* can be translated into improved productive performance, which could be of particular values to the post-weaned pig. Rooke *et al*. (1996) studied the use of soybean meal, pretreated with protease, in diets for weaned pigs and showed clear benefits in growth performance in the critical first 7 days after weaning (Figure 2.4). Similarly, Beal *et al*., (1998b), working with both raw and autoclaved soybeans in wet-fed diets for growing pigs (32 kg star weight), also saw improvement in performance following protease addition (Figure 2.5).

It was clear that the enzyme hydrolyses the soybean protein into lower molecular weight units (Beal *et al*., 1998c), coupled with its effects on the proteinaceous anti-nutrients (trypsin inhibitors and lectin), offered interesting possibility for the future. Added-values soya based products using enzyme technology are now already available on the feed industry, offering good opportunities for more cost-effective piglet diet formulation. Equally, with the increasing interesting in wet feeding systems for different weight classed of pigs in some areas of the world, a process involving enzymatic pre-digestion of certain components prior to diet mixing also becomes feasible.

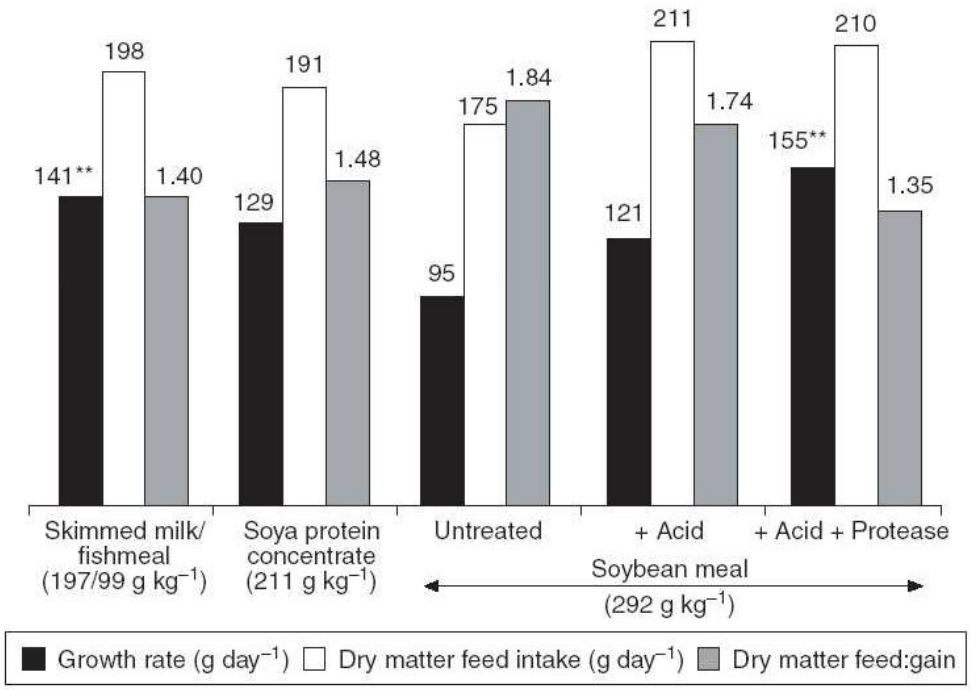


Figure 2.4 The effect of pretreatment of soybean meal with acid **–/ +** protease on piglets performance in the first 7 days after weaning (Rooke *at al*., 1996).

Soybean meal treated for 3 h at 50°C, pH 4.5 and 20% dry matter; products neutralized and then dried (65°C) before inclusion into a maize diet + amino acids + vitamin/minerals, lysine 1.2%, digesible energy 14.5 MJ kg^{-1} (3465 kcal). ** P<0.01.

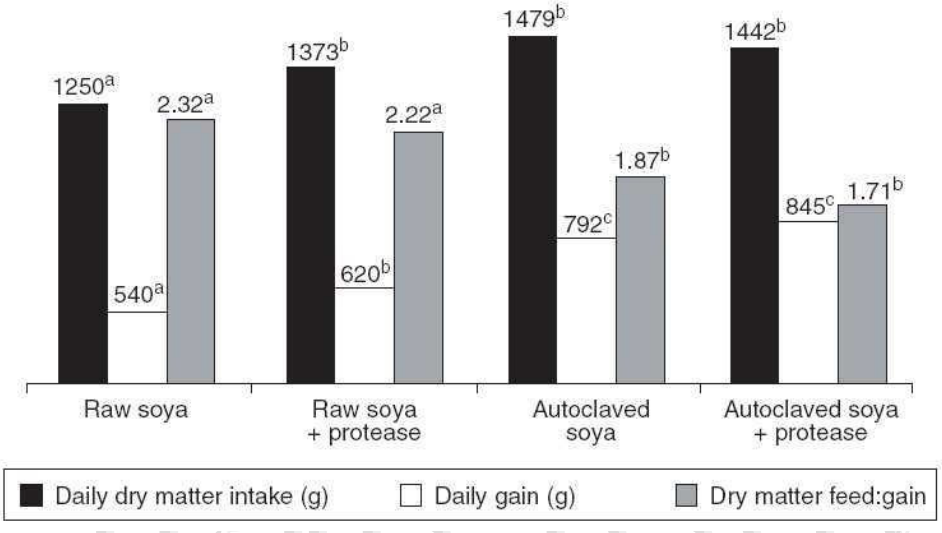


Figure 2.5 The effect of protease pretreatment of raw or autoclave full fat soybeans on grower pig performance (32 kg start weight) (Beal *et al*., 1998b).

Mean values with different superscripts (a,b) in the same parameters indicate significant differences (P<0.05).

2.5 *Bacillus* sp.

The genus *Bacillus* consists of a large number of diverse, rod-shaped, gram positive (or positive only in early stages of growth) bacteria that are motile by peritrichous flagella and are aerobic. They are capable of producing endospores that are highly resistant to unfavorable environment conditions (Claus and Berkeley, 1986). The majority of *Bacillus* are mesophiles, with temperature optima between 30 and 45°C, but the genus also contains a number of thermophilic species with optima as high as 65°C. This genus consists of a diverse group of organisms as evidenced by the wide range of DNA base ratios of approximately 32 to 69 mol% G + C (Hoch and Losick, 1993), which is far wider than that usually considered reasonable for a genus (Norris et al., 1981). Most *Bacillus* species are versatile chemoheterotrophs capable of respiration using a variety of simple organic compounds (sugars, amino acids, organic acids). They produces a variety of proteases and other enzymes that enable it to degrade a variety of natural substrates and contribute to nutrient cycling. Furthermore, *Bacillus* is considered a benign organism as it does not possess traits that cause disease. It is not considered pathogenic or toxigenic to humans, animals, or plants. The potential risk associated with the use of this bacterium in fermentation facilities is low.

2.6 Protease production

2.6.1 Liquid fermentation (LF)

The production of bacterial protease is undertaken with a variety of cultivation systems ranging from sub-merged and solid substrate system to immobilized cell technique (Beshay, 2003; Tunga *et al*., 1999; Woufrs, and Buysman, 1977). The enzyme is mainly produced, however, by batch fermentation in stirred tank fermentation. Proteases are generally produced by submerged fermentation which higher production than solid state fermentation processes (George *et al*., 1995; Chakraborty, 1993; Malathi and Chakraborty, 1991). Increased in the production in microbial culture can be archived by a suitable metabolic regime (such as by feed strategy) and optimization of the relationship between the microorganism and the environment parameters include the type of fermentation, concentration of nutrient, dissolved oxygen tension, agitator type and speed, pH control, and temperature.

2.6.2 Solid state fermentation (SSF)

A solid state fermentation is a process which involves the growth of microorganisms on porous solid substrates in the absence of free water. Most solid state fermentations use fungi, because they are excellent producers of extracellular hydrolases that break up the biopolymers in the solid substrates, and they are capable of colonising and invading solid particles, i.e. they do not have to be mixed homogeneously through the substrate in order to achieve efficient utilisation of all the substrate. Table 2.8 shows advantages and disadvantages of SSF compared to LF.

Table 2.8 Comparison between liquid and solid substrate fermentations (Raimbault, 1998).

Factor	Liquid Substrate Fermentation	Solid Substrate Fermentation
Substrates	Soluble substrates (sugars)	Polymer insoluble substrates: starch cellulose pectines lignin
Metabolic Heating	Easy control of temperature	Low heat transfer capacity
pH control	Easy pH control	Buffered solid substrates
Mechanical agitation	Good homogeneization	Static conditions prefered
Scale up	Industrial equipments available	Need for Engineering & New design Equipment
Inoculation	Easy inoculation , continuous process	Spore inoculation, batch
Energetic consideration	High energy consuming	Low energy consuming
Volume of Equipment	High volumes and high cost technology	Low volumes &low costs of equipments
Effluent and pollution	High volumes of polluting effluents	No effluents, less pollution

2.7 Protease

Proteases execute a large variety of complex physiological functions. Their importedance in conducting the essential metabolic and regulatory functions is evident from their occurrence in all forms of living organisms. Proteases play a critical role in many physiological and pathological processes including protein catabolism, blood coagulation, control of cell growth and differentiation, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens, release of hormones and pharmacologically active peptides from precursor proteins, and transport of secretory proteins across membranes (Suzuki et al., 1997). In general, extracellular proteases catalyze the hydrolysis of large proteins to smaller molecules for subsequent absorption by the cell whereas intracellular proteases play a critical role in the regulation of metabolism. In contrast to the multitude of the roles contemplated for proteases, our knowledge about the mechanisms by which they perform these functions is very limited.

The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey and West, 1996). Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications.

2.7.1 Bacterial protease

Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) and have relatively low thermotolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than do the animal proteinases and hence are valuable for use in the food industry. Neutrase, a neutral protease, is insensitive to the natural plant proteinase inhibitors and is therefore useful in the brewing industry. The bacterial

neutral proteases are characterized by their high affinity for hydrophobic amino acid pairs. Their low thermotolerance is advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis. Some of the neutral proteases belong to the metalloprotease type and require divalent metal ions for their activity, while others are serine proteinases, which are not affected by chelating agents. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry.

2.7.2 Fungal protease

Fungi elaborate a wider variety of enzymes than do bacteria. For example, *Aspergillus oryzae* produces acid, neutral, and alkaline proteases. The fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity. However, they have a lower reaction rate and worse heat tolerance than the bacterial enzymes. Fungal enzymes can be conveniently produced in a solid-state fermentation process. Fungal acid proteases have an optimal pH between 4 and 4.5 and are stable between pH 2.5 and 6.0. They are particularly useful in the cheesemaking industry. Fungal neutral proteases are metalloproteases that are active at pH 7.0 and are inhibited by chelating agents. In view of the accompanying peptidase activity and their specific function in hydrolyzing hydrophobic amino acid bonds, fungal neutral proteases supplement the action of plant, animal, and bacterial proteases in reducing the bitterness of food protein hydrolysates. Fungal alkaline proteases are also used in food protein modification.

2.7.3 Classification of protease

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in class 3 (The hydrolase) and subclasss 3.4 (The peptide hydrolase or peptidase) (International Union of Biochemistry, 1992). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently, proteases are classified on the basis of three major criteria: (i) type of reaction

catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure (Barett, 1994).

Proteases are subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds from the termini of the substrate. Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960)

2.7.3.1 Exopeptidases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino- and carboxypeptidases, respectively.

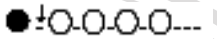
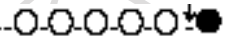
Aminopeptidases

Aminopeptidases act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide (Table 2.8). They are known to remove the N-terminal Met that may be found in heterologously expressed proteins but not in many naturally occurring mature proteins. Aminopeptidases occur in a wide variety of microbial species including bacteria and fungi (Watson, 1976).

Carboxypeptidases

The carboxypeptidases act at C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases, metallocarboxypeptidases, and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of the enzyme.

Table 2.8 Classification of proteases (Rao *et al*., 1998).

Protease	Mode of action [a]	E.C. no.
Exopeptidases		
Aminopeptidases		3.4.11
Dipeptidyl peptidase		3.4.14
Tripeptidyl peptidase		3.4.14
Carboxypeptidase		3.4.16-3.4.18
Serine type protease		3.4.16
Metalloprotease		3.4.17
Cysteine type protease		3.4.18
Peptidyl dipeptidase		3.4.15
Dipeptidases		3.4.13
Omega peptidases		3.4.19
		3.4.19
Endopeptidases		3.4.21-3.4.24
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metalloprotease		3.4.24
Endopeptidases of unknown catalytic mechanism		3.4.99

[a] Open circles represent the amino acid residues in the polypeptide chain. Solid circles indicate the terminal amino acids, and stars signify the blocked termini. Arrows show the sites of action of the enzyme.

2.7.3.2 Endopeptidases

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. The presence of the free amino or carboxyl group has a negative influence on enzyme activity. The endopeptidases are divided into four subgroups based on their catalytic mechanism, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine proteases, and (iv) metalloproteases.

Serine proteases

Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to the organisms. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. Based on their structural similarities, serine proteases have been grouped into 20 families, which have been further subdivided into about six clans with common ancestors (Barett, 1994).

Serine proteases are recognized by their irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DCI), L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E-64), diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Some of the serine proteases are inhibited by thiol reagents such as p-chloromercuribenzoate (PCMB) due to the presence of a cysteine residue near the active site. Serine proteases are generally active at neutral and alkaline pH, with an optimum between pH 7 and 11.

(i) Serine alkaline proteases

Serine alkaline proteases are produced by several bacteria, molds, yeasts, and fungi. They are inhibited by DFP or a potato protease inhibitor but not by tosyl-L-phenylalanine chloromethyl ketone (TPCK) or TLCK. Their substrate specificity is similar to but less stringent than that of chymotrypsin. They hydrolyze a peptide bond which has tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. The optimal pH of alkfaline proteases is around pH 10, and their isoelectric point is around pH 9. Their molecular masses are in the range of 15 to 30

kDa. Although alkaline serine proteases are produced by several bacteria such as *Arthrobacter*, *Streptomyces*, and *Flavobacterium* spp. (Boguslawski *et al*., 1983), subtilisins produced by *Bacillus* spp. are the best known. Alkaline proteases are also produced by *S. cerevisiae* (Mizuno and Matsuo, 1984) and filamentous fungi such as *Conidiobolus* spp. (Pearl and Taylor, 1987) and *Aspergillus* and *Neurospora* spp. (Lindberg *et al*., 1981).

(ii) Subtilisins

Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, subtilisin Carlsberg and subtilisin Novo or bacterial protease Nagase (BPN9), have been identified. Subtilisin Carlsberg produced by *Bacillus* licheniformis was discovered in 1947 by Linderstrom, Lang, and Ottesen at the Carlsberg laboratory. Subtilisin Novo or BPN9 is produced by *Bacillus amyloliquefaciens*. Subtilisin Carlsberg is widely used in detergents. Its annual production amounts to about 500 tons of pure enzyme protein. Subtilisin BPN9 is less commercially important. Both subtilisins have a molecular mass of 27.5 kDa but differ from each other by 58 amino acids. They have similar properties such as an optimal temperature of 60°C and an optimal pH of 10.

Aspartic proteases

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin (A1), retropepsin (A2), and enzymes from pararetroviruses (A3) (Barett 1995). Most aspartic proteases show maximal activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5. The aspartic proteases are inhibited by pepstatin (Fitzgerald *et al*., 1990). They are also sensitive to diazoketone compounds such as diazoacetyl-DL-norleucine methyl ester (DAN) and 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP) in the presence of copper ions. Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin. Microbial aspartic proteases can be broadly divided into two groups, (i) pepsin-like

enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus,* and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor* spp.

Cysteine/thiol proteases

Cysteine proteases occur in both prokaryotes and eukaryotes. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsin- like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv) others. Papain is the best-known cysteine protease. Cysteine proteases have neutral pH optima, although a few of them, e.g., lysosomal proteases, are maximally active at acidic pH. They are susceptible to sulfhydryl agents such as PCMB but are unaffected by DFP and metal-chelating agents.

Metalloproteases

Metalloproteases are the most diverse of the catalytic types of proteases (Barett, 1995). They are characterized by the requirement for a divalent metal ion for their activity. They include enzymes from a variety of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria (Shannon *et al*., 1989; Wilhelm, *et al*., 1987; Okada *et al*., 1986; Hibbs *et al*., 1985 and Weaver et al., 1977). Based on the specificity of their action, metalloproteases can be divided into four groups, (i) neutral, (ii) alkaline, (iii) Myxobacter I, and (iv) Myxobacter II. The neutral proteases show specificity for hydrophobic amino acids, while the alkaline proteases possess a very broad specificity. Myxobacter protease I is specific for small amino acid residues on either side of the cleavage bond, whereas protease II is specific for lysine residue on the amino side of the peptide bond. All of them are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP.

(i) Collagenase

Another important metalloprotease, was first discovered in the broth of the anaerobic bacterium *Clostridium hystolyticum* as a component of toxic products. Later, it was found to be produced by the aerobic bacterium *Achromobacter iophagus* and other microorganisms including fungi. The action of collagenase is very specific; i.e., it acts only on collagen and gelatin and not on any of the other usual protein substrates. Elastase produced by *Pseudomonas aeruginosa* is another importedant member of the neutral metalloprotease family.

(ii) Thermolysin

Thermolysin produced by B. stearothermophilus is a single peptide without disulfide bridges and has a molecular mass of 34 kDa. It contains an essential Zn atom embedded in a cleft formed between two folded lobes of the protein and four Ca atoms which impart thermostability to the protein. Thermolysin is a very stable protease, with a half-life of 1 h at 80°C.

In summary, proteases are broadly classified as endo- or exoenzymes on the basis of their site of action on protein substrates. They are further categorized as serine proteases, aspartic proteases, cysteine proteases, or metalloproteases depending on their catalytic mechanism. They are also classified into different families and class depending on their amino acid sequences and evolutionary relationships. Based on the pH of their optimal activity, they are also referred to as acidic, neutral, or alkaline proteases.