POTENTIAL USE OF PROTEASE ENZYMES IN LIQUID DIETS FOR PIGS

by

JANE DAVINA BEAL

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ABSTRACT

JANE DAVINA BEAL

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A programme of study was undertaken to assess the effect of pretreating raw soyabean and processed full fat soyabean meals with protease enzymes prior to use in liquid feed for grower and finisher pigs.

A series of laboratory studies was undertaken to examine the efficacy of three microbial proteases (P2, P3 and P4) in partially hydrolysing soya protein and in reducing the levels of trypsin inhibitors in raw soyabean. Pretreatment consisted of steeping ground soyabean for 24 h at 20° C in the absence (control) or presence of 20 000 units g^{-1} N of P2, P3 or P4. Pretreating raw soyabean (RSB) with P2, P3 and P4 significantly ($P < 0.05$) reduced trypsin inhibitor levels from 28.53 to 19.98, 17.17 and 18.35 (s.e.m.1.14) mg trypsin inhibited g^{-1} soya respectively. Pretreating RSB, micronized (MIC) or autoclaved (AUT) soyabean meal with P2, P3 or P4 resulted in increases in soluble α-amino nitrogen of 5.22, 7.08, and 6.58 (RSB), 5.11, 5.57 and 4.32 (MIC) and 3.56, 7.03 and 6.18 (s.e.d. 0.06) mg g^{-1} soya respectively and *in vitro* digestibility of nitrogen of 7.6 %, 9.9 % and 6.4 % (RSB), 4.9 %, 8.3 % and 2.8 % (MIC) and 11 %, 8 % and 12.2 % (AUT) respectively compared with the appropriate controls.

Feeding trials were conducted in which pretreated soya was added to a basal cereal diet. Pretreatment of RSB with P4 resulted in a significant ($P < 0.05$) increase in ADG of 0.08 kg pig^{-1} d^{-1} (s.e.d. 0.04) in grower pigs (33.5 ± 4 kg) over the 6 week duration of the trial but had no significant effect on FCR. Pretreatment of AUT with P4 did not significantly improve performance. In a feeding trial with grower/finisher pigs pretreatment of RSB with P3 resulted in significant ($P < 0.05$) improvements of 0.10 (s.e.d. 0.04) kg pig^{-1} d^{-1} in ADG and 0.476 (s.e.d. 0.19) in FCR. Pretreatment of MIC with P3 resulted in a significant reduction ($P < 0.05$) of 4 d (s.e.d. 1.7) in the time taken for pigs to attain slaughter weight.

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External Contacts.

Dr. H. Schulze, Finnfeeds International, Marlborough UK.
Dr. H. Simmins, Finnfeeds International, Marlborough UK.
Mr. D.Wiggins, Metabolic Research Laboratory, Oxford University, Oxford UK.
Dr. B. Miller, Bristol Veterinary School, Langford, Bristol UK
Miss J. Thorpe, Bristol Veterinary School, Langford, Bristol UK
Dr. P Hobbs, Institute of Grassland and Environmental Research, Devon, UK.

signed.....Jane Beal.....
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ABBREVIATIONS

α-AN	α-amino nitrogen
ADFI	average daily feed intake
ADG	average daily gain
ANF	Antinutritional factor
AUT	Autoclaved full fat soyabean meal
BBI	Bowman- Burk trypsin/chymotrypsin inhibitor
CCK	Cholecystokynin
FCR	feed conversion ratio
IVDN	*in vitro* digestibility of nitrogen
KTI	Kunitz trypsin inhibitor
MIC	Micronized full fat soyabean meal
NSP	Non starch polysaccharide
RSB	Raw soyabean
SBM	Soyabean meal
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPC	Steam pressure cooked full fat soyabean meal
SPI	Serine protease inhibitor
TSD	Toasted full fat soyabean meal

Chapter 1

Literature Review.

1.1. Introduction

Almost all biochemical reactions occurring in living organisms are mediated by enzymes. This large diverse group of proteins possess a number of common properties that enable them to fulfil their role as biological catalysts. They have a high calalytic power, typically increasing reaction rates by factors of 10^6 to 10^{12} (Voet and Voet 1995). They catalyse reactions under mild conditions, namely atmospheric pressure and physiological ranges of temperature and pH. They have a high degree of substrate and product specificity and have the capacity to be regulated as their proteinaceous nature imparts a precise 3 dimensional structure allowing the formation of specific binding sites for substrates and/or regulatory effectors.

Enzymes show varying degrees of specificity which generally depends on their biological role. The least specific enzymes are often involved in degradative processes and exhibit bond specificity. Such enzymes interact with a type of chemical bond, for example a peptide bond or a glycosidic bond, and will catalyse the breakdown of substrates containing that specific bond. Enzymes exhibiting group specificity have ranges of substrates that are restricted to those possessing a specific chemical group for example a phosphate group or an acyl coA group. Absolute specificity on the other hand limits the enzyme to a single specific substrate or specific pair of substrates. The latter two types of enzyme are typical of those involved in intermediary metabolism and biosynthesis (Price and Stevens 1991).

Enzymes have been used in human food production, albeit inadvertently, through fermentation processes for thousands of years (Campbell-Platt and Cook 1991). The recognition in the last 100 years or so of enzymes as the 'active' constituents of these fermentation processes has resulted in the establishment of a branch of the food industry concerned with enzymology (Price and Stevens 1991). Consequently, the application of exogenous enzymes to modify food substances is well established in the human food industry, e.g. the inversion of sucrose by invertase or the coagulation of milk protein by rennin (Feeney and Whitaker 1985). These enzymatic modifications often take place under controlled conditions in food processing plants, with applications of well defined enzymes and substrates resulting in a consistent end product.

The use of exogenous enzymes as animal feed additives has received much attention over the past few decades. The potential for improving the utilization of feedstuffs by supplementation with exogenous enzymes may be summarized as follows:

- to counteract deficiencies in the digestive enzyme capacity of young animals by supplementation with digestive enzyme analogues such as proteases or amylases
- to act as an extension of the digestive capacity of an animal by supplementation with enzymes cabable of degrading substrates that the animal does not produce enzymes to digest. For example β-glucanase or xylanase to degrade plant cell walls
- to target specific antinutritional factors such as phytic acid and phytates to reduce the detrimental effects associated with these substrates.

Comprehensive reviews of the use of enzymes in animal feeds have appeared in the literature over the last five years (Campbell and Bedford 1992; Chesson 1993; Johnson, Williams and Campbell 1993; Dierick and Decuypere 1994; Graham and Balnave 1995; Bedford and Schulze 1998). These authors generally agree that enzyme supplementation of

animal feeds has, in the past, been a rather *ad hoc* process in which crude enzyme extracts were often added to whole diets either as a pre-mix or prior to pelleting. This was possibly due to a lack of commercial enzyme preparations specifically developed for the animal feed industry (Perry 1995). Certainly the controlled enzymatic processes used in the human food industry are not apparent in the application of enzymes to animal feeds, although all of these authors highlight the need to target specific substrates under defined conditions.

The majority of enzymes used in animal feeds are applied to the dry diet, often prior to processing treatments such as pelleting. Much of the attention of the feed additive manufacturers has been focused on producing enzymes or enzyme preparations and delivery systems that will withstand feed processing treatments. However, most of the enzymes currently of interest to the animal feed industry such as β-glucanase, amylase, proteases and phytase suffer temperature related reduction in activity due to heat treatments (Gadient 1993). Phytase activity has been shown to be reduced by ~50% and ~ 75% at pelleting temperatures of 75°C and 80°C respectively (Simoes Nunes 1993). This is not suprising because the activity of an enzyme is a function of its 3 dimensional structure, and any conformational changes that affect the active site will affect activity. Extremes of heat irreversibly denature enzymes, with the temperature at which denaturation occurs depending on the enzyme in question (Voet and Voet 1995). As most commercial enzyme preparations are derived from micro-organisms this tends to reflect the temperature tolerance of the organism from which the enzyme is derived. Enzymes from mesophilic organisms often rapidly loose activity at temperatures above 40° C whereas those from thermophiles can remain active at higher temperatures (Price and Stevens 1991).

Another parameter that can have important consequences for enzyme activity is the availability of water. For most enzymes an aqueous environment is necessary for activity

as water is an important component in maintaining enzyme structure as well as enabling substrates and products to diffuse to and from the active site. For hydrolases water is also required as a reactant (Price and Stevens 1991). Water activity (a_w), a measure of equilibrium relative humidity, is often used in the food industry to define water available for microbial growth. Although some enzymes of microbial origin are capable of activity at a_w levels below those required for microbial growth, at least a monomolecular layer of water must be present around the enzyme (Campbell-Platt and Cook 1991). In rehydration of dried lysozyme preparations this was achieved when the water content reached 38% (Price and Stevens 1991). The percentage of water in a material cannot be directly correlated to a_w because the latter measures available water, i.e. water that has not interacted with any substrate, whereas the former will measure water that is loosely bound to a substrate and not necessarily available to support microbial growth or enzyme activity. The a_w of dried cereals is around 0.65 (Prescott, Harley and Klein 1993) with a water content of around 12% (NRC 1998). No data are available of the a_w requirements of enzymes commonly used in animal feeds, however, work with other enzymes indicate that water contents greater than 20% are needed to initiate any appreciable enzyme activity (Price and Stevens 1991). Undoubtedly one of the main problems encountered in the application of enzyme pretreatments to commercially produced compound feed and feed mixtures is the inability of enzymes to act efficiently in the dry environment provided by these media.

The addition of enzymes to dry diets implies that any exogenous enzyme activity must occur in the gut of the animal after ingestion of the feed. In the case of the pig it is unlikely that sufficient rehydration of the feed would occur in the oesophagus, therefore, exogenous enzymes must be able to survive the low pH environment prevailing in the stomach and resist attack by proteolytic digestive enzymes. Denaturation of enzymes by changes in pH

are often reversible. At low pH conformational changes in enzyme structure are brought about by the increasing H^+ concentration disrupting H-bonds and electrostatic bonds between amino acid residues that maintain the enzyme's 3 dimensional shape. However, if pH is restored to the optimum for that enzyme the active conformation is often restored (Voet and Voet 1995). The ability of enzymes to survive exposure to low pH varies between different enzymes. For example β-glucanase appears to be more susceptible to pH denaturation than pentosanase (Baas and Thacker 1996; Thacker and Baas 1996). These authors showed that in the duodenal digesta of pigs fed diets supplemented with these enzymes 84% of pentosanase activity was recovered 4 hours after feeding compared with 26% of β-glucanase activity.

An alternative approach would be to pretreat individual raw materials with exogenous enzymes in an aqueous environment to obtain a degree of substrate degradation prior to feeding. Liquid feeding presents an ideal opportunity to adopt this approach. On many UK farms liquid feed systems for pigs are used to enable farmers to take advantage of liquid by-products from food processors, thus diets are often mixed on the farm (Geary 1997). It is not too difficult to envisage a situation where pretreatment of an individual raw material with an exogenous enzyme could be undertaken *in situ* prior to mixing with the other dietary components.

1.2 The current commercial use of enzymes in animal nutrition

The current commercial application of enzymes to monogastric animal diets centres around the non-starch polysaccharide (NSP) degrading enzymes, β-glucanase, xylanase and pentosanase. In broiler diets the use of these enzymes to improve the nutritional status of one or more dietary components is well established. It is estimated that the current addition of enzymes to poultry feeds is worth £26 million per annum to the UK broiler industry (Perry 1995). Some of the success of enzyme additions to poultry diets would appear to be due to the ability of these enzymes to degrade NSP of plant cell walls. Inclusion of dietary ingredients such as barley into poultry diets causes an increase in digesta viscosity which results in sticky droppings and has a detrimental effect on feed conversion ratio (Pluske and Lindemann 1998) due to the presence of the NSP β-glucan. The mode of action of these enzymes in poultry is generally accepted as being a decrease in digesta viscosity resulting in a reduction in sticky droppings and an increase in performance when added to barley or rye based diets (Classen and Bedford 1991; Campbell and Bedford 1992; Thomke and Elwinger 1998). Some of the data presented in these reviews is summarized in Table 1.2.1. Although increases in performance have been achieved with pigs fed barley or rye diets supplemented with NSP degrading enzymes, their application has had less of an impact in the pig production industry. This appears to be partly because the pig does not suffer from digestive disturbances due to the inclusion of NSP in the diet (Campbell and Bedford 1992). The limited response of pigs to NSP inclusion in the diet may be related to species differences in the structure and physiology of the gastrointestinal tract. In poultry food is held for approximately 2 hours in the non-secretory crop at a pH of 6 - 6.5 allowing exogenous enzymes time to act, whereas in the pig the time taken for ingested food to reach the stomach may be measured in minutes (Dierick and Decuypere 1994; Schulze 1995). In addition, the residence time of food in the stomach and small intestine of the pig is much

longer (4 - 5 hours) than the residence time in poultry (1 - 2 hours) (Dierick and Decuypere 1994). The dry matter content of the food as it passes along the small intestine is much greater in the chick, with solute concentrations being as much as twice those experienced by the pig (Bedford and Schulze 1998). The gut microflora of poultry has less of an impact on digestion than that of the pig. For instance, there is evidence that many lactobacilli inhabiting the pig intestine have the ability to degrade β-glucan linkages in barley endosperm cell walls (Chesson 1993). Digesta viscosity appears to be of less consequence in the pig, partly due to increased residence time in the stomach and partly due to the larger gut capacity of the pig and the fermentation of fibre in the hindgut (Dierick and Decuypere 1994). In support of this, Lewis, McEvoy and McCracken (1998) found no correlation between *in vitro* wheat viscosity and ileal digestibility of dry matter, crude protein, fibre or energy of pig diets. While β-glucanases and pentosanases degrade the cell walls of cereals such as barley and rye they may not be as efficient in degrading cell walls of legume seeds as these tend to contain hemicellulose, pectin and oligosaccharides such as stachyose and raffinose (Charlton 1996). Therefore, different enzyme preparations may be needed to target these feed ingredients.

Recent attention has focused on the use of mixtures of enzymes in pig diets. For example in wheat/barley based diets addition of Porzyme tp100 (Finnfeeds International), a mixture of xylanase, β-glucanase, amylase and pectinase, improved both ADG and FCR by 4 % (Anon. 1993). In barley based diets a mixture of β-glucanase, α-amylase and glucoamylase improved ADG and FCR by 8.9% and 2.5% respectively (Thomke and Elwinger 1998). An enzyme complex, (Allzyme vegpro, Alltech inc.), with the main activity provided by a protease, and designed to improve the nutritional value of legumes improved both ADG and FCR in grower/finisher pigs fed corn-soyabean meal diets by approximately 7% (Lindemann 1997).

Table 1.2.1. Percentage increases (over control) in growth rate and feed conversion of pigs and poultry fed enzyme supplemented barley or rye based diets.

Animal	Age/Weight	Diet base	Enzyme supplement	% increase growth rate	% increase FCR
pig	11 -15 kg	rye	xylanase	0	1.4
	12 - 18 kg	rye - barley	β-glucanase	17.1	12.9
	9 - 14 kg	barley	β-glucanase	3.3	4.2
	20 - 98 kg	rye	pentosanase	6.4	2.0
	20 - 85 kg	rye	β-glucanase	-4.0	-3.3
	20 -85 kg	barley	β-glucanase	2.4	2.4
chicken	0 - 21 days	barley	Roxazyme	4.6	4.3
	1 - 44 days	barley	Roxazyme	3.4	4.4
	0 - 21 days	barley	Roxazyme	2.1	4.4
	laying hens	barley	Roxazyme	2.4	1.0
	broiler cockerels*	rye	xylanase	34.4	15.1
	broiler cockerels*	wheat	xylanase	1.6	0

* data from Classen and Bedford (1991) all other data from Thomke and Elwinger (1998)
Roxazyme is a multi-enzyme complex containing β-glucanase, cellulase and amylase

A considerable amount of work has been done on the addition of phytases to animal feedstuffs. The addition of phytase increases the availability of phosphorus and some other inorganic cations by the hydrolysation of natural phytates and phytic acid, the storage compounds for phosphorus in most plants. This reduces the need to add inorganic phosphorus to the diet and consequently reduces phosphorus output in the effluent. This approach is becoming more attractive in some parts of Europe with the imposition of phosphorus output levies (Perry 1995). The use of phytase in animal feeds was reviewed by Bedford and Schulze (1998). It appears that variations observed in animal responses to phytase inclusion in diets depends not only on the animal species targeted but also on its age and physiological status. In poultry the efficacy of phytase is greater in older birds

compared with chicks. In pigs the responses to phytase inclusion in the diet also vary. Sows in the mid-term of pregnancy appear to require far greater levels of phytase supplementation to replace 1g of inorganic phosphorus than lactating sows or grower/finisher pigs (Bedford and Schulze 1998). These authors also infer that comparisons between different trials may not necessarily be valid unless other dietary factors such as the levels of calcium or cholecalciferol, which influence the efficacy of phytase supplementation, are taken into account.

To date little work has been undertaken on the addition of enzymes to pig diets to improve the nutritional status of some of the other nutrients of plant origin. It has been recognized that there is considerable potential for the pretreatment of dietary components such as soyabean (Classen, Balnave and Bedford 1993). Soyabeans provide a high quality protein source in pig diets that is relatively well digested with an apparent ileal digestibility of lysine of 81 % (NRC, 1998). However, due to the ubiquitous use of soyabean in animal feed even small improvements in N digestibility could give cost benefits. Soyabeans require heat treatment and processing to achieve this level of digestibility due to the presence of heat labile antinutritional factors (ANF). Many of the factors that affect the utilization of raw soyabeans by monogastric animals are proteinaceous in nature. Several authors of papers dealing with ANFs and the application of enzymes in animal feeds have postulated that exogenous proteases could be used to reduce the activity of ANF's such as the serine protease inhibitors and antigenic proteins in raw soyabean (Huisman and Tolman 1992; Classen *et al.* 1993; Morgan and Mul 1993; Dierick and Decuypere 1994).

1.3 Soyabeans in animal feed

1.3.1 *Soyabeans as a protein source*

Soyabeans, *Glycine max.* a member of the Leguminosae, originated in eastern Asia and have been cultivated in China since about 3000 B.C, being first mentioned in Chinese literature in 2838 B.C. They were introduced into the US at the turn of the century, and commercial processing of oil and protein was established by 1922. Soyabean meal, a by-product of oil processing, has been used as an animal feed since the 1930's (Smith and Circle 1978), and in more recent years full fat soya has been increasingly used in animal feeds.

The soyabean seed consists of the following definable structures, the hull, the cotyledon and the hypocotyl and plumule, which contribute 8%, 90% and 2% of the total weight of the seed respectively. The hull which surrounds the cotyledon consists of an outer layer of palisade cells, followed by consecutive definable layers of hourglass cells, small compressed parenchyma cells, aleurone cells and compressed layers of endosperm. The cotyledon consists of an outer epidermal layer covering densely packed elongated palisade-like parenchyma cells. The parenchyma cells of the cotyledon represent the main storage organ of the seed and contain protein bodies and spherosomes (Waggle and Kolar 1978). The majority of soya protein is stored in the protein bodies which vary in size between 2 and 20 μm in diameter and are surrounded by a unit membrane (Martinez 1978). Interspersed between them in the cytoplasm of cotyledon cells are smaller spherosomes (0.2 -0.5μm diameter) which store oil.

The main storage proteins of soyabeans are glycinin and β-conglycinin, which together represent approximately 90% of the storage protein. Other proteins that are present in appreciable amounts are a diverse group of serine protease inhibitors (SPI) and lectins.

These together make up 6 - 7 % of the total protein, the remainder being functional proteins such as enzymes and cell membrane proteins (Nielsen 1983). The solubility of soya protein in water is pH dependant and is at a minimum between pH 3.75 and pH 5.25 due to the isoelectric points of the main protein constituents occurring at these pH values (Pearson 1983). Ultracentrifugation profiles of water extractable (pH 7) proteins reveal four major fractions based on sedimentation values (Svedberg units), 2S, 7S, 11S and 15S. Whilst each fraction contains a complex mix of proteins certain components can be identified. The 2S fraction (~20% extractable protein) contains the serine protease inhibitors and a number of metabolic proteins. The 7S and 11S fractions, each comprising ~33% of extractable protein consist mainly of β-conglycinin and glycinin respectively. The 15S fraction has not been studied extensively but may consist of aggregated 11S protein (Nielsen 1985). β-conglycinin is a multimeric globular glycoprotein with a molecular mass of 105 - 193 kDa. It consists of three subunits, is low in methionine and cystine and hence sulphur (0.6%) and contains ~5% of the sugars glucose and mannose. Glycinin is a multimeric globular protein with a molecular mass of 309 - 393 kDa and consists of six subunits. It has higher levels of methionine and cystine than β-conglycinin and contains 1.8% sulphur (Nielsen 1983).

The physiological role of storage proteins in the plant is to provide a source of reduced nitrogen for germination and seedling growth. Legume storage proteins tend to contain relatively high proportions of the amide amino acids glutamine and asparagine, which is consistent with this role. The amino acid profiles of raw soyabean, soyabean meal, some of the common vegetable protein sources for animal feeds and fish meal, calculated by Yin, Huang, Zhang, Chen and Pan (1993) in a comprehensive study of the nutritive values of a range of 38 commonly used feedstuffs, are presented in Table 1.3.1. Although it is

deficient in the sulphur containing amino acids methionine and cystine, soya protein is the most balanced of the plant protein sources commonly used in animal feeds (Spencer 1983).

In Table 1.3.2 the essential amino acid balance of these protein sources are compared to the ideal amino acid balance for growing pigs as based on the concept of the 'ideal protein' (Cole 1996). The values given in these tables are representative values and, as with all biological material, there will be some degree of variation. The data in Table 1.3.2 shows that, while an animal protein source such as fish meal contains sufficient of all the essential amino acids, plant proteins are generally deficient in one or more. Soya protein is deficient in lysine, leucine and the sulphur amino acids. Even so, the main protein components of animal feeds world-wide are supplied by vegetable protein, and the main source of that protein comes from the soyabean (Huisman and Tolman 1992). The nutritional value of a protein depends not only on the amino acid composition but also on how well the proteins are digested, absorbed and utilized by the animal.

In 1917 Osborne and Mendel reported a low nutritional value for raw soya and a high nutritional value for cooked soya when fed to rats (Smith and Circle 1978). It was apparent that soyabeans contained heat labile substances that interfered with the digestion and/or absorption of protein. These substances became generally known as antinutritional factors, and were defined by Chubb (1982) as

> those substances generated in natural feedstuffs by the normal metabolism of the species from which the material originates and which by different mechanisms exert effects contrary to optimum nutrition.

This definition could be interpreted to include fibre, which is not generally regarded as an antinutritional factor although high inclusion levels can have detrimental effects on nutrient

absorption in pigs (English, Fowler, Baxter and Smith 1996). A less cumbersome definition was supplied by Huisman (1992), namely,

> Antinutritional factors can be defined as non-fibrous natural substances having negative effects on growth or health of man or animals.

In this definition fibre is excluded as well as artificial antagonists introduced in processing or by contamination by pesticide, herbicides, chemical additives or mycotoxins.

In the 1940's world shortages of protein prompted research into the nutritional value of soya protein for animal feeds and the effects of processing treatments. This led to the discovery of the serine protease inhibitors by Ham, Sandstedt, and Mussehl (1945). These are the major antinutritional factors in soyabeans, although a number of other ANF's have been identified, for example, lectins, phytic acid, saponins and tannins (Liener 1994). Only the proteinaceous antinutritional factors in soyabeans will be dealt with in this review.

Table 1.3.1. A comparison of the amino acid composition of some plant protein sources commonly used in animal feeds and fish meal.

	Amino acid composition (g kg^{-1} dry matter)					
Amino acid	Raw soya	Soya bean meal	Rape seed meal	Cottonseed meal	Groundnut meal	Fish meal
Alanine	15.8	23.4	17.3	18	27.6	42.6
Arginine	17.8	35.1	19.8	48.6	76.6	43.4
Aspartic acid	30.6	52	25.1	39.8	67.7	69.8
Cystine		6.9	8.4	7.1	6.2	7.3
Glutamine	45.5	85.5	63.9	89.2	114.9	100
Glycine	16.4	20.2	18.9	19.4	34	47.8
Histidine	7.4	12.2	18.5	12.8	15.4	18.9
Isoleucine	14.8	19.7	18.6	12.5	19.4	29.1
Leucine	21.2	33.2	29.7	25.2	31.9	49.3
Lysine	20.4	28.9	17.7	15.8	20.5	51.7
Methionine	2	3.4	7.9	5.6	4.7	14.4
Phenylalanine	14.6	23.5	25.7	21.5	23	28.8
Proline	16.9	26.4	21.3		4.9	8.3
Serine	14.4	23.2	13.5	16.3	25.7	23.8
Threonine	11.8	19.3	16.1	14.4	16.3	29.5
Tryptophan	4.9	6.1	1.9	5.2	3.3	7.5
Tyrosine	7.4	13.6	16.1	8.6	14.6	15.3
Valine	17.4	23.9	25.1	21.7	15.7	40.9

Adapted from Yin *et al.* (1993)

Table 1.3.2. Essential amino acid composition of some commonly used protein sources in animal feeds compared with that of the 'ideal protein' for a growing pig

	Amino acid composition (g kg^{-1} protein)						
Amino acid	Ideal protein [1]	Raw soyabean[2]	Soyabean meal [2]	Fish meal[2]	Rapeseed meal[2]	Cottonseed meal[2]	Groundnut meal[2]
Lysine	70.0	49.5	56.6	81.7	41.3	40.2	41.0
Methionine + cysteine	35.0	4.9*	20.2	35.3	38.0	32.3	21.8
Tryptophan	10.0	11.9	11.9	11.8	4.4	13.2	6.6
Threonine	42.0	28.6	35.8	46.6	37.5	36.6	32.6
Leucine	70.0	51.5	55.0	77.9	69.2	64.1	63.8
Valine	49.0	42.2	46.8	64.6	58.5	55.2	31.4
Isoleucine	38.0	35.9	38.6	46.0	43.4	31.8	38.8
Phenylalanine + tyrosine	67.0	53.4	62.6	69.7	97.4	76.6	75.2
Histidine	23.0	18.0	23.9	29.9	43.0	32.6	30.8
Non-essential amino acids	596.0	704.1	648.8	537.6	567.1	617.3	658.0

* no figures were available for the cysteine content of raw soya giving a false value for met + cyst
Source - [1] McDonald, Edwards, Greenhalgh and Morgan 1996 [2] Calculated from Yin *et al.* (1993)

1.3.2. *Antinutritional factors.*

1.3.2.1 *The serine protease inhibitors.*

The serine proteases are a group of enzymes so called because they have a common catalytic mechanism that involves a highly reactive serine residue. The pancreatic enzymes trypsin, chymotrypsin and elastase belong to this group. They all catalyse the hydrolysis of peptide bonds but differ in specificity. Trypsin cleaves peptide bonds adjacent to arginine or lysine residues, chymotrypsin cleaves bonds adjacent to phenylalanine, tryptophan or tyrosine residues and elastase cleaves bonds adjacent to small neutral residues such as alanine. The substrate specificity of serine proteases is dictated by the sequence of amino acid residues in a pocket shaped binding site on the enzyme (Voet and Voet 1995). The catalytic mechanism of the pancreatic serine proteases involves the association of the substrate and enzyme to form an enzyme - substrate complex which is held together by interactions between the side chain of the scissile peptide and the binding pocket of the enzyme. The hydroxyl group of the highly reactive serine residue reacts with the carbonyl group of the peptide, cleaving the bond and in doing so forming a tetrahedral intermediate. The tetrahedral intermediate collapses forming an acyl-enzyme complex and in doing so releases the amine product. Subsequent hydrolysation of the acyl-enzyme product releases the active enzyme and the carboxyl product (Fersht 1985; Voet and Voet 1995).

Naturally occurring inhibitors of serine proteases are widespread in plants, animals and micro-organisms. They have an important regulatory role in animals but their physiological function in plants is uncertain. It is generally supposed that their role is one of protection against attack by insects, micro-organisms and other pests (Norton 1991). Protease inhibitors are classified into families according to their source (animal, plant or microbe) and mechanism of action. The serine protease inhibitors of soyabean belong to

two of these families, the soyabean trypsin inhibitor (Kunitz) family and the soyabean proteinase inhibitor (Bowman- Birk) family (Laskowski 1986).

In the seed the soyabean Kunitz inhibitor is localized in cell walls, protein bodies, cytoplasm and nuclei of the cells of the cotyledon and embryonic axis, but is not present in the hypocotyl (Horisberger and Tacchini-Vonlanthen 1983). The inhibitor was first crystallized and characterized by Kunitz in the 1940's and the complete amino acid sequence and secondary structure (shown in Figure 1.3.1) was discovered by Koide *et al.* in 1973 (Norton 1991). Several isoinhibitors of this family are found in soyabeans. They have a molecular weight of around 20kDa with two disulphide bridges, and exhibit inhibitory action against trypsin only. There is a high degree of homology between isoinhibitors. They all consist of approximately 180 amino acid residues with only minor differences between them. In each case the active site consists of an arginine and an isoleucine residue situated at positions 63 and 64 respectively (Liener and Kakade 1980; Laskowski 1986).

The inhibitory action of the Kunitz trypsin inhibitor (KTI) of soyabeans has been thoroughly investigated. KTI acts as a competitive inhibitor. The arginine 63 residue of its reactive site binds to the binding pocket of trypsin forming a highly stable complex. X-ray crystallographic studies of the enzyme inhibitor complex of KTI reveal that only 12 of the 181 amino acid residues of KTI are involved in complex formation. These residues, which include the reactive site peptide bond between Arg-63 and Ile-64, are highly complementary with the binding pocket of the enzyme enabling the formation of closer than Van-der-Waals contact which gives the complex stability (Laskowski 1986). These studies also show that the reactive site peptide bond remains intact in the complex (Laskowski 1986; Norton 1991) and therefore there is no acyl-enzyme formation. Recent

work by Vaintraub and Haram (1995) on the proteolytic degradation of purified KTI shows that a high degree of proteolysis is required before there is a significant reduction in inhibitory activity. This suggests that the 12 amino acid residues involved in complex formation retain activity when other sections of the protein are cleaved.

Protease inhibitors of the Bowman-Birk family are localized in the protein bodies nuclei and cytoplasm of the cells of the cotyledon and embryonic axis of the soyabean seed. In contrast to the Kunitz inhibitor the Bowman-Birk inhibitor (BBI) is also found in the intercellular spaces but not in the cell walls (Horisberger and Tacchini-Vonlanthen 1983). Inhibitors of this family have typical molecular weights of around 8 kDa with seven disulphide bridges and exhibit inhibitory action against both trypsin and chymotrypsin. The amino acid sequence and covalent structure of soyabean BBI was determined by Odani and Ikenaka (1973), (Figure 1.3.2) and consists of 71 amino acid residues with two active sites one for trypsin and one for chymotrypsin (Norton 1991). The trypsin and chymotrypsin active sites are located at Lys16 - Ser17 and Leu 43 - Ser 44 respectively and each is situated in a loop of nine peptides formed by a single disulphide bond. BBI forms a 1:1 complex with either trypsin or chymotrypsin and a ternary complex with both. Complex formation has been postulated as similar to that of KTI for both trypsin and chymotrypsin but the scissile bond in each case may be cleaved but the amine product constrained within the binding pocket preventing the formation of the acyl-enzyme (Norton 1991). The cleavage of the scissile bond results in a modified inhibitor which has similar activity to the unmodified inhibitor, and the two exist in dynamic equilibrium attached to the binding site of the enzyme.

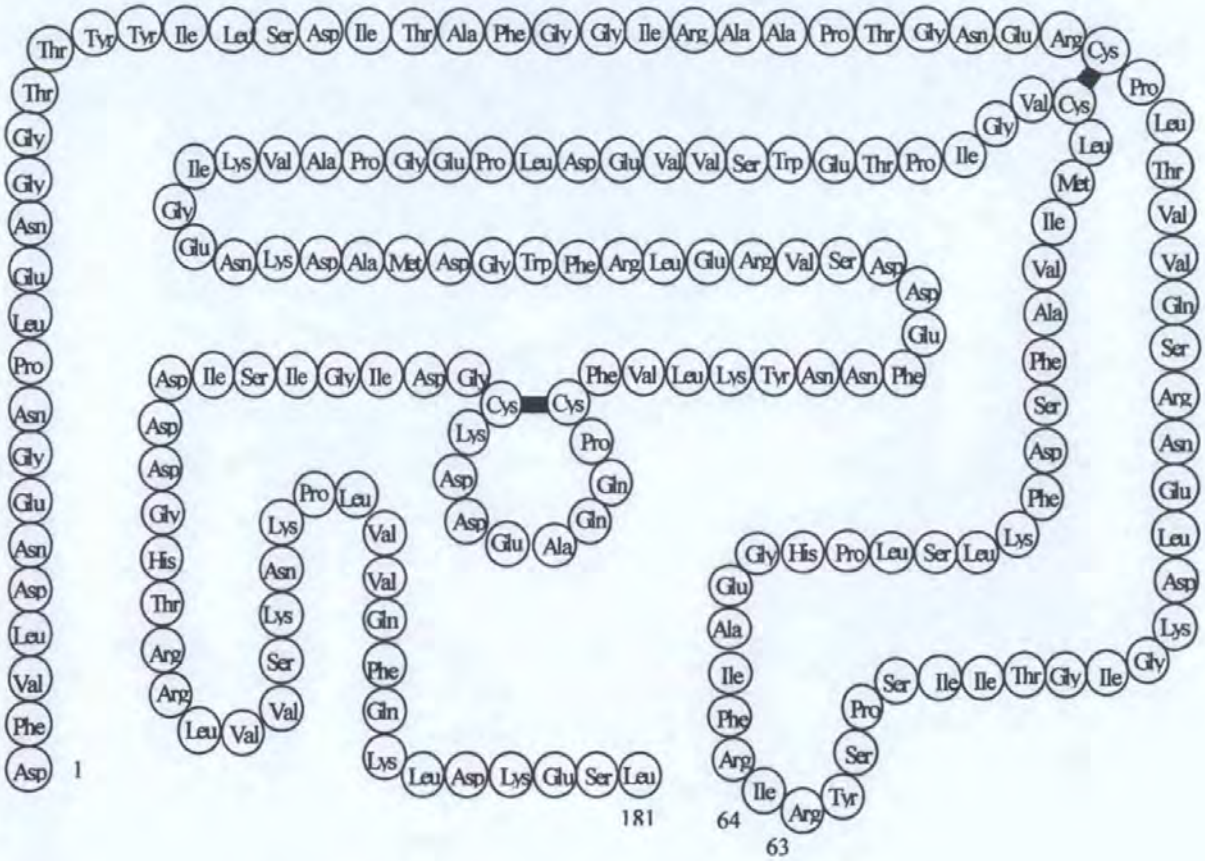


Figure 1.3.1. Structure and amino acid sequence of the Kunitz soya bean trypsin inhibitor. (From Koide *et al.* 1973)

Figure 1.3.2. Structure and Amino acid Sequence of the Bowman-Birk Inhibitor

(from Odani and Ikenaka 1973)

1.3.2.2. *Lectins.*

Lectins are heat labile antinutritional factors and their presence in soyabeans may account for at least some of the growth inhibitory ability of raw soyabeans from which the protease inhibitors have been removed (Jaffe 1980). Lectins are glycoproteins and have the ability selectively to bind to carbohydrate residues of cell membrane glycoproteins. Lectins from different sources have differing affinities for carbohydrate residues. The soyabean lectin is a tetrameric glycoprotein with a molecular weight of about 120 kDa. Each tetramer has 4 binding sites specific for N-acetyl-D-galactosamine (Liener 1994). It has been estimated that approximately 60% of ingested lectin survives transit through the stomach and binds to N-acetyl-D-galactosamine residues present on the apical membranes of enterocytes, disrupting the brush border and causing atrophy of the microvilli. Consequently, there is a reduction in epithelial cell viability and hyperplasia of the crypt cells leading to poor nutrient absorption, and an increase in epithelial cell turnover and mucus secretion. These effects again lead to an increase in endogenous nitrogen loss and poor performance (Classen *et al.* 1993). Ingestion of soyabean lectins have also been implicated in lowering insulin levels in the blood, interfering with the absorption of lipids and iron from the diet and inhibition of some digestive enzymes (Liener 1994). The carbohydrate residues of apical membrane glycoproteins in the enterocytes of pigs and some other species change with age. Therefore the antinutritive effects of lectins tend to be age related as well as species specific (Classen *et al.* 1993). Most of the work on the antinutritional effects of soyabean lectins has been conducted in rats and the effects extrapolated to other species. There appears to be no direct evidence that soyabean lectins are toxic to pigs at normal dietary inclusion levels (Jaffe 1980; Huisman and Tolman 1992).

1.3.3. *The physiological effects of raw soyabeans in animal diets.*

Extensive reviews of the physiological effects of ingesting serine protease inhibitors have appeared in the literature in the last 15 years or so (Liener and Kakade 1980; Chubb 1982; Rackis, Wolf and Baker 1986; Rothman 1986; Birk 1989; Norton 1991; Huisman and Tolman 1992; Gueguen, Vanoort, Quillien and Hessing 1993; Le Guen and Birk 1993; Van Kempen 1993; Liener 1994). While most of the work has been carried out on rats and chicks, other species; guinea pigs, calves, pigs, geese, monkeys and dogs, have been investigated but nowhere near as thoroughly. It is obvious from the literature that there are differences in responses to inclusion of protease inhibitors in the diet both between species and for animals of different age or health status within a species. Ingestion of serine protease inhibitors either in raw soyabean or as a purified inhibitor results in growth depression in young and growing animals of all species studied. However, the degree of growth depression is dependant on a number of factors including the age of the animal, the content and quality of the protein in the diet and the presence of other antinutritional factors (Schneeman and Gallaher 1986).

Liener (1949) demonstrated that the growth depression associated with the ingestion of serine protease inhibitors was not simply due to the inhibition of protein digestion by formation of a stable trypsin /SPI complex. Diets containing trypsin inhibitors and pre-digested protein and free amino acids were still capable of inhibiting the growth of rats. It is generally accepted that growth depression is accompanied by pancreatic hypertrophy in rats and chicks, but not in larger animal species such as pigs, dogs and calves. It appears that species in which the weight of the pancreas exceeds 0.3 % of the body weight, such as rats, are more susceptible to pancreatic hypertrophy in response to ingestion of raw soyabean (Liener and Kakade 1980). Studies with rats have shown that ingestion of serine protease inhibitors interfere with a feedback mechanism in the small intestine whereby a

fall in trypsin and chymotrypsin activity stimulates the release of a hormone, cholecystokinin (CCK), from the jejunal endocrine cells which in turn stimulates the pancreas to produce and secrete more enzymes. This hypersecretion of pancreatic enzymes results in endogenous nitrogen losses and accounts for the observed pancreatic hypertrophy and partly accounts for the growth depression (Liener 1994). Due to the high cystine content of the serine proteases and relatively low sulphur amino acid content of soya based diets this may result in a deficiency of sulphur amino acids which may also partly account for poor growth. Indeed the addition of methionine or cystine to raw soyabean meal resulted in protein utilization close to that of heat treated soyabean meal in rats and chicks (Liener and Kakade 1980).

The effects of feeding raw soyabean meals to pigs have been investigated by a number of workers (Jiminez, Perry, Pickett and Beeson 1963; Combs, Conness, Berry and Wallace 1967; Young 1967; Hanke, Rust, Meade and Hanson 1972; Yen, Hymowitz and Jensen 1974; Yen, Jensen and Simon 1977; Vandergrift, Knabe, Tanksley and Anderson 1983; Crenshaw and Danielson 1985b; Pontif, Southern, Coombs, McMillin, Bidner and Watkins 1987; Cook, Jensen, Fraley and Hymowitz 1988; Hancock, Peo, Lewis, Chiba and Crenshaw 1990; Herkelman, Cromwell, Stahly, Pfeiffer and Knabe 1992; Friesen, Nelssen, Goodband, Behnke and Kats 1993). All of the above conducted feeding trials in which growth responses of pigs to diets containing raw soyabean meals (RSB) or processed soyabean meals (SBM) were compared. The results of these studies are summarized in Table 1.3.3. The data compiled in this table highlights the difficulty in drawing direct comparisons between results obtained in different studies which have used different feed regimes and experimental design and animals of different ages. However, some pertinent points can be extracted from these data. All authors reported reduced growth performance of pigs fed RSB. Of the authors that practized *ad libitum* feeding on their trials, Crenshaw

Table 1.3.3. Summary of growth responses of pigs to diets containing raw soyabean

pig weight / age	feed regime	raw soya		processed soya		type of soya/ inclusion level in diet	reference
		ADG (kg)	FCR	ADG (kg)	FCR		
15 kg		0.45	3.97	0.84	3.08	27% RSB *v* 27% SBM (+ 0.25% methionine both diets)	Jiminez *et al.* 1963
8 weeks		0.19	4.38	0.64	1.99	28% RSB 21% *v* SBM	Combs *et al.* 1967
				0.50	2.26	28% RSB 28% *v* 28% heat treated soya 120°C 30min.	
2 weeks		0.21	3.01	.34	2.35	17% RSB + 5% fat supplement *v* 28% SBM	
		0.20	2.85			22% RSB + 5% fat supplement *v* 28% SBM	
22 kg	*ad lib.*	0.39	3.87	0.62	2.74	22.15% RSB *v* 17.85% SBM	Young 1967
50 kg	*ad lib.*	0.52	4.55	0.83	3.34	15.15% RSB *v* 12.2% SBM	
57.5 kg		0.572	4.0	0.781	3.22	15.7% RSB *v* 11.6% SBM (48% CP)	Hanke et al. 1972
11 kg	*ad lib.*	0.22	3.125	0.51	1.88	37.3% LT RSB *v* 32 % SBM	Yen *et al.* 1974
11 kg	*ad lib.*	0.19	3.125			39.8% LT RSB *v* 32 % SBM	
12 kg	*ad lib.*	-0.05		0.16	3.0	42.5% RSB *v* 32% SBM	Yen *et al.* 1977
12 kg	*ad lib.*	0.04	8.0	.51	1.94	42.5% RSB *v* 32% SBM	
25 -45 kg	meal	0.04	16.6	0.36	2.85	23% defatted RSBF *v* 23% heated (100 °C/25 min) SBF	Vandergrift *et al.* 1983
				0.31	3.45	23% defatted RSBF *v* 23% heated (107 °C/105 min)SBF	
23 kg	*ad lib.*	0.37	4.70	0.74	3.23	33.5% RSB *v* 26.2% SBM	Crenshaw *et al.* 1985
45 kg	*ad lib.*	0.57	4.65	0.88	3.56	26.7% RSB *v* 20.5% SBM	
68 kg	*ad lib.*	0.56	5.25	0.83	4.05	19% RSB *v* 14.8% SBM	

Table 1.3.3. continued

pig weight / age	feed regime	raw soya		processed soya		type of soya/ inclusion level in diet	reference
		ADG kg	FCR	ADG kg	FCR		
59 - 62 kg	*ad lib.*	0.696	3.95	0.825	3.62	19.7% RSB *v* 15.8% SBM	Pontif *et al.* 1987
7 kg	*ad lib.*	0.286	2.89	0.436	1.98	32 5% LT RSB *v* 26% SBM	Cook *et al.* 1988
	ad lib.	0.211	4.23			32 5% HT RSB *v* 26% SBM	
67 kg	*ad lib.*	0.87	3.412	0.95	3.436	18% LT RSB *v* 14% SBM	
	ad lib.	0.83	3.70			18% HT RSB *v* 14% SBM	
6.7 kg	*ad lib.*	0.015	12.5	.185	2.33	defatted RSB F *v* autoclaved (20min.) defatted RSB F	Hancock *et al.*1990
24 kg	meal	0.295	4.89	414	3.45	25% HT RSB *v* 25% heated (110°C/20 min) HT RSB	Herkelman *et al.*
	meal	0.322	4.45	459	3.14	25% LT RSB *v* 25% heated (110°C/20 min) LT RSB	1992
5.8kg	*ad lib.*	0.071	3.57	0.222	1.30	45% defatted RSBF *v* 45% extruded RSBF	Friesen *et al.* 1993

RSB raw soyabean, HT RSB high (i.e. conventional) trypsin inhibitor raw soyabean, LT RSB low trypsin inhibitor raw soyabean,
SBM defatted (solvent extracted) heat treated soyabean meal - 44% crude protein unless otherwise specified

and Danielson (1985b), Cook *et al.*(1988), Friesen *et al.* (1993), Yen *et al.* (1974), Yen *et al.* (1977) and Hancock *et al.* (1990), observed reduced feed intake in pigs fed RSB compared to SBM. Whilst reduction in feed intake is undoubtedly a factor in retardation of growth in these pigs it does not appear to be the whole story. Vandergrift *et al.* (1983) restricted feed intake of all pigs to that of pigs fed RSB diets and observed an 89% reduction in average daily gain (ADG) in ileal cannulated pigs fed RSB compared with SBM, whilst Herkelman (1992) using a similar feed regime observed 28.7% reduction in ADG in pigs fed RSB. Viewing the results of these trials overall it would appear that pigs fed RSB in their diets fare better when allowed *ad libitum* access to their food. However, the responses of pigs to RSB in particular, but also to SBM in respect of average daily gain and feed conversion ratios varies considerably between studies.

The susceptibility of pigs to antinutritional factors in raw soyabean does appear to be age dependant. Both Crenshaw and Danielson (1985b) and Cook *et al.* (1988) observed greater percentage differences in response to RSB in younger pigs. The percentage reduction in ADG and percentage increases in feed conversion ratios (FCR) are summarized in Table 1.3.4. The lessening of the detrimental effects of RSB in the diet with increasing age of the pig is supported by the work of Crenshaw and Danielson (1985a) and Yen *et al.* (1991). Crenshaw and Danielson (1985a) fed RSB to gestating sows for three parities and reported no adverse effect on subsequent litter size or piglet survival when compared with sows fed SBM. They also found that RSB fed during gestation did not interfere with gestation weight gain or lactation weight loss. During this study sows were fed conventional SBM diets during lactation. In a later study Yen *et al.* (1991) reported similar findings for gestation weight gain and first litter size. However, if RSB was fed to sows during lactation, this resulted in greater lactational weight loss due to decreased feed intake when compared to SBM. It has also been reported that egg production in laying hens is not

affected by the inclusion of RSB in the diet (Liener and Kakade 1980). These studies suggest that mature animals of these species are not adversely affected by ANF's in raw soyabeans.

Table 1.3.4 Percentage (of control) responses of pigs of different age/weight to RSB diets compared with SBM diets.

Initial weight of pig (kg)	% decrease in ADG	% increase in FCR	reference
7	51 (HT)	113.6 (HT)	Cook *et al.* 1988
7	34 (LT)	30.8 (LT)	Cook *et al.* 1988
23	50	45	Crenshaw and Danielson 1985
45	35	30.6	Crenshaw and Danielson 1985
67	12.6 (HT)	7.4 (HT)	Cook *et al.* 1988
67	8.4 (LT)	+1.4 (LT)	Cook *et al.* 1988
68	32	29.6	Crenshaw and Danielson 1985

HT - high trypsin inhibitor raw soya bean; LT - low trypsin inhibitor raw soyabean

Yen *et al.* (1974), Cook *et al.* (1988), Vandergrift *et al.* (1983) and Hancock *et al.* (1990) conducted nitrogen balance studies on pigs fed either RSB or SBM. They all observed a reduction in retained N in pigs fed RSB diets. Table 1.3.5 summarizes their results. Yen *et al.* (1974) also reported differences in N retention between young gilts and hogs in response to RSB. Hogs retained 50% N and gilts 41% N when fed an RSB diet compared with 59% N (hogs) and 63% N (gilts) for SBM. This suggests young gilts are more susceptible to the ANF's in RSB than young hogs.

Table 1.3.5. Summary of nitrogen balance studies on pigs fed RSB diets compared with SBM diets.

pig weight (kg)	% nitrogen retained		reference
	RSB diet	SBM diet	
14	31 (HT)	58	Yen *et al.* 1974
	23 (LT)		
12.4	50.9 (HT)	65.9	Cook *et al.* 1988
	59.5 (LT)		
25 - 45	37.0	60.5	Vandergrift *et al.* 1983
17.2	37.3	51.0	Hancock *et al.* 1990

HT - high trypsin inhibitor (conventional) raw soya bean;
LT - low trypsin inhibitor raw soyabean

Vandergrift *et al.* (1983), Li *et al.* (1998) and Herkelman *et al.* (1992) determined the apparent ileal digestibilities of protein and amino acids in pigs fed RSB diets compared with SBM diets. The former two authors also determined faecal digestibilities. They all found that the digestibility of protein and all of the amino acids, both essential and non essential, were lower in pigs fed RSB. Their results for protein and lysine digestibility are summarized in Table 1.3.6. Work by Hagemeister and Barth (1993) supports these findings, and indicates that the poor response of pigs to RSB diets is due to increased endogenous N loss rather than malabsorption of exogenous N. They found the apparent ileal disappearance of N to be 5.7 ± 16.9 % for RSB and 54 ± 5% for SBM., and the true ileal disappearance of N to be 92 ±1.5% for RSB and 96.4 ± 0.4% for SBM.

Some studies have been undertaken in which a proportion of SBM in the diet was replaced by RSB. When 25% SBM was replaced with RSB, N retention (% of intake) was reduced from 53.5% to 41.4% (Grala, Verstegen, Jansman, Huisman and Wasilewko 1998b). Ileal crude protein and lysine digestibilities were also reduced from 82.8% to 68.1% and 90.6% to 80.3% respectively (Grala, Verstegen, Jansman, Huisman and van Leeuwen 1998a).

Young (1967) observed significant reductions in ADG of grower pigs when RSB replaced 50%, 75% or 100% of SBM in the diet but not when it replaced 25% of SBM in the diet. FCR on the other hand was only significantly depressed at 75% and 100% inclusion levels. In contrast the ADG and FCR of finisher pigs was significantly depressed only when RSB provided 100% supplemental protein in the diet. Walker *et al.* (1987) found no significant differences in ADG, ADFI or FCR when 10% or 20% of SBM in the diet was substituted with RSB in diets for grower pigs, but a significant depression in these parameters was detected when RSB replaced 40% SBM in the diet. Likewise Pontif *et al.* (1987) observed reduced growth performance in pigs fed diets in which RSB provided 33% or 67% of the protein. The data from these trials is summarized in Table 1.3.7.

Table 1.3.6. Summary of apparent ileal and faecal digestibilities of RSB and SBM

	ileal digestibility %		faecal digestibility %		
Item	RSB	SBM	RSB	SBM	reference
Crude protein	37.4	77.1	77.3	90.2	Li *et al.* 1998
Nitrogen	35.1	78.9	71.2	88.6	Vandergrift *et al.* 1983
Nitrogen	57.5(HT)	74.4			Herkelman *et al.* 1992
Nitrogen	60.0(LT)	74.8			Herkelman *et al.* 1992
Lysine	40.8	79.6	79.6	90.2	Li *et al.* 1998
Lysine	44.2	84.9	71.9	87.3	Vandergrift *et al.* 1983
Lysine	59.8(HT)	70.5			Herkelman *et al.* 1992
Lysine	66.9(LT)	77.4			Herkelman *et al.* 1992

HT - high trypsin inhibitor (conventional) raw soya bean;
LT - low trypsin inhibitor raw soyabean

Table 1.3.7. Summary of results of trials in which RSB was substituted for SBM and provided a proportion of the protein in the diet

% replacement of SBM with RSB	ADG	FCR	Reference
0 (a)	0.62	2.74	Young 1967
25	0.62	2.96	
50	0.50	3.06	
75	0.45	3.64	
100	0.39	3.87	
0 (b)	0.83	3.33	
25	0.86	3.27	
50	0.79	3.35	
75	0.72	3.51	
100	0.52	4.55	
0 (c)	0.85	3.05	Walker *et al.* 1987
10	0.81	3.13	
20	0.82	3.03	
40	0.75	3.21	
0 (d)	0.825	3.62	Pontif *et al.* 1987
33	0.754	3.60	
67	0.763	3.73	
100	0.696	3.95	

Initial weight of pigs: (a) 22 kg; (b) 50 kg; (c) 18 kg; (d) 57.2 kg

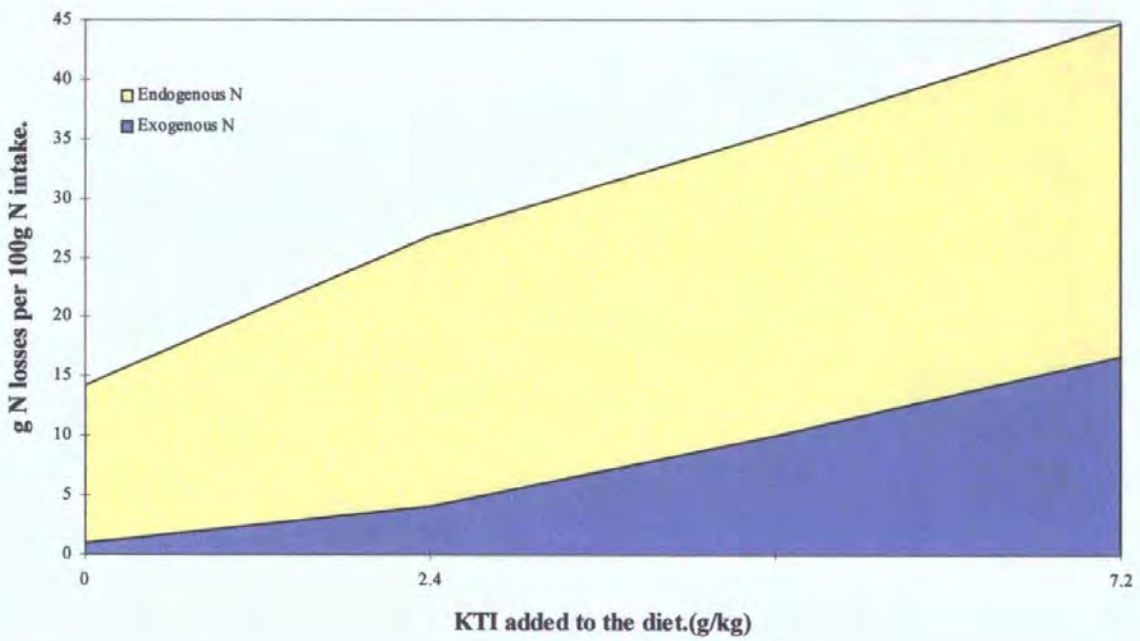
The poor response of pigs fed diets containing RSB is generally attributed to the presence of serine protease inhibitors. The studies undertaken by Yen *et al.* (1974), Cook *et al.* (1988) and Herkelman *et al.* (1992) indicate that certainly some of the growth retardation experienced by pigs on RSB diets is due to the presence of trypsin inhibitors. These workers used varieties of soyabean with low trypsin inhibitor activity (LT) compared with varieties with normal levels of trypsin inhibitor activity (HT). Tables 1.3.3, 1.3.4. and 1.3.5. include data from these studies. LT RSB resulted in increased performance compared with HT RSB, but in most cases performance was reduced when compared to diets containing SBM. However, in comparing soyabeans with different trypsin inhibitor activities to conventional processed soyabean meals care must be taken in apportioning cause, as a number of other factors may obscure the issue. Genetic variants that express

different trypsin inhibitor activities may also express differences in other proteins and ANF's. Of the papers reviewed here, Combs *et al.* (1967), Vandergrift *et al.* (1983), Hancock *et al.* (1990), Herkelman *et al.* (1992), Friesen *et al.* (1993), and Li *et al.* (1998) stated that the SBM's used in their studies came from the same batch as the RSB. Other authors used unspecified commercial soyabean meals.

A clearer picture of the effect of trypsin inhibitors on the performance of pigs can be obtained from work by Yen *et al.* (1977) and Schulze *et al.* (1993a) on the effect of isolated soyabean Kunitz trypsin inhibitors added to SBM diets. Both of these studies revealed no differences in pancreas weight between pigs fed diets containing KTI and those fed diets without KTI. Also KTI consumption decreased trypsin activity in both the pancreas and small intestine. In this respect the physiological response of pigs to serine protease inhibitors appears todiffer from that of rats in which the inclusion of KTI in the diet results in hypersecretion of pancreatic enzymes. The evidence from these studies suggests that the CCK feedback mechanism, that increases pancreatic secretion of trypsin and chymotrypsin in response to low intestinal activities of these enzymes, does not respond to dietary inclusion of serine protease inhibitors in pigs in the same way that it does in rats. However, the CCK mediated feed back system depends on both ileal trypsin and ileal chymotrypsin activity. Schulze *et al.* (1993b) fed pigs diets containing isolated KTI at inclusion levels of 0, 2.4 and 7.2 g KTI kg^{-1} diet. They found that ileal trypsin activities fell from 167.8 in the control diet to 89.5 units with the 2.4g KTI diet but chymotrypsin activity actually increased from 55 to 145 units respectively. Therefore, pancreatic secretion may not have been stimulated at low levels of KTI inclusion due to high ileal chymotrypsin activities. However, in the 7.2g KTI diet both ileal trypsin and chymotrypsin activities fell, to 3 and 21.3 units respectively, which should have increased pancreatic secretion of these enzymes. Schulze *et al.* (1993a) also measured endogenous and exogenous N losses in response to

increasing levels of KTI in the diet. Their results support those of Hagemeister and Barth (1993), namely that it is the loss of endogenous N rather than the failure to absorb exogenous N that has a detrimental effect on the growth performance in pigs. In the study of Schulze *et al.* (1993a) apparent ileal N digestibilities of 85.8 %, 73.1 % and 55.3 % were obtained for diets containing 0, 2.4 and 7.2g kg^{-1} KTI in the diet respectively, with respective values for true ileal N digestibility of 99.1 %, 95.9 % and 83.3 %. The relative contribution of endogenous and exogenous N in the ileal digesta is represented in Figure 1.3.1 Increasing KTI levels above 2.5 g kg^{-1} significantly decreased exogenous N absorption as well as increasing endogenous N loss.

Fig 1.3.3. Dose response of growing pigs to dietary inclusion of isolated Kunitz Trypsin inhibitor showing relative proportions of endogenous and exogenous nitrogen loss.



After Schulze *et al.* 1993

Schulze *et al.* (1993b) proposed the following mechanism for the inhibition of growth performance and increased N losses in these pigs. As KTI levels increase, more trypsin is complexed with the inhibitor in the gut. KTI/trypsin complex formation interferes with

protein digestion and prevents reabsorbtion of the enzyme. The low pancreatic trypsin and chymotrypsin activities observed may be accounted for by the fact that these enzymes have a high cystine content. Therefore, the continuous loss of cystine via enzyme inhibitor complex formation reduces the sulphur amino acids available to the animal (as cystine can be derived from methionine). This may not only result in reduced production of cystine-rich enzymes in the pancreas but also in reduced growth due to S-amino acid deficiency. The situation would be further exacerbated by the low S-amino acid content of soyabean. However, this is not the complete picture because, if the above explanation were the case, supplementation of high trypsin inhibitor diets with S-amino acids should result in improved growth performance in pigs as it does in rats and chicks. Yet both Jiminez *et al.* (1963) and Yen *et al.* (1977) observed that supplementing RSB diets with methionine did not result in increased growth performance compared with unsupplemented RSB diets. No data are available on the response of pigs fed isolated KTI diets to supplemental methionine. Therefore, the lack of response could have been due to the presence of ANF's other than KTI in the raw soyabean.

Native legume storage proteins, including those of soyabean, have been described as being inherently resistant to mammalian digestive enzymes (Nielsen, Deshphande, Hermodson and Scott 1988) possibly due to intrinsic structural factors (Carbonaro, Cappelloni, Nicoli, Lucarini and Carnovale 1997). Nielsen *et al.* (1988) demonstrated that glycinin and β-conglycinin were more readily degraded by trypsin and chymotrypsin after heat treatment. Soya proteins have also been implicated in causing allergenic reactions in susceptible animals (Stokes, Moller, Bailey, Wilson and Bourne 1987; Lalles, Salmon, Bakker and Tolman 1993; Dreau, Lalles, Chevaleyre, Toullec and Salmon 1993). Having said that, the serine protease inhibitors are generally perceived as the major antinutritional factors in raw soyabean.

1.3.4. *Processing and heat treatments of soyabean.*

It is well established that adequately processed soyabeans are more digestible by monogastric animals than raw or inadequately processed soyabeans (Huisman and Tolman 1992). Adequate processing usually involves some form of heat treatment. Heat treatments denature proteins and it is generally accepted that the heat denaturation of proteinaceous ANF's is the main contributory factor in improving the digestibility of soyabeans (Melcion and van der Poel 1993; Wareham, Wiseman and Cole 1994). Heat denaturation of the serine proteases, KTI and BBI, destroys the integrity of their 3 dimensional structure, the maintenance of which is fundamental to their inhibitory properties. Heat treatments not only denature ANF's, but also affect storage proteins and there is evidence that these may behave differently in the whole soya protein matrix than they do in isolation. For example, isolated glycinin forms insoluble aggregates upon heating, whereas heat induced interactions between the subunits of glycinin and β-conglycinin include the formation of soluble macro-complexes, (Damodaran and Kinsella 1982). Storage proteins may also be involved in heat inactivation of BBI through disulphide interchanges disrupting the integrity of the active site (Melcion and van der Poel 1993).

Commercial heat processing is carefully controlled. Underheating can result in inadequate inactivation of ANF's whilst overheating may reduce amino acid availability through the occurrence of Maillard reactions, in which lysine residues become crosslinked to reducing sugars. The availability of methionine, leucine and isoleucine may also be reduced following overheating (Nesheim and Carpenter 1967). The common commercial processes for soyabeans are outlined in Table 1.3.8. The efficiency with which heat treatments reduce ANF's to acceptable levels depends on a combination of temperature, heating time, initial water content and the amount of water added during the process. Any

additional physical process such as milling or flaking will also have an effect (Melcion and van der Poel 1993).

A number of studies have been undertaken on the effect of various processing methods on the nutritive value of soyabeans (Noland, Campbell, Gage, Sharp and Johnson 1976; Clawson, Ramsey and Armstrong 1981; Rudolph, Boggs, Knabe, Tanksley and Anderson 1983; Vandergrift *et al.* 1983; Walker, Maxwell, Owens and Buchanan 1986; Knabe, LaRue, Gregg, Martinez and Tanksley 1989; Friesen *et al.* 1993; Marty, Chavez and de Lange 1994; Sohn, Maxwell, Southern and Buchanan 1994; Grala *et al.* 1998). Most of these studies conclude that no single processing treatment is superior to another, but that all processed soya beans are nutritionally superior to raw soya (Rackis *et al.* 1986; Melcion and van der Poel 1993). A number of these authors have determined apparent ileal digestibilities of N for some commercially processed soyabean products. Their results are summarized in Table 1.3.9. Qin *et al.* (1996) compared different temperature/time combinations of steaming on the ileal digestibility of full fat soyabeans. They found no significant difference in ileal digestibility between soyabeans treated at 102°C /40min, 120° C/7.5 min or 134 °C/1.5min, whereas with soyabeans treated at lower temperatures or for shorter times digestibility was significantly reduced. These results are summarized in Table 1.3.10., along with those of Herkelman *et al.* (1992) and Li *et al.* (1998) who also determined apparent ileal digestibilities of soyabeans that had been heat-treated under similar temperature/time regimes.

Table 1.3.8. Commercial methods for processing soyabeans

Process	Conditions		Heat source	Action
	Temperature (°C)	Time (min.)		
Extrusion	80 - 100	0.5 - 2.5	steam/ electricity	HTST - raw material forced through a die by one or more screws generating shear and frictional forces, additional heat supplied by steam
Steam explosion	140 - 210	0.3 - 0.75	steam/ gas	HTST - raw material steamed under high pressure, sudden release of pressure causes rapid expansion
Micronisation	80 - 130	0.6 - 1.0	infra red	HTST - raw material passed under an infra red heater, molecules vibrate at resonsant frequencies causing rapid internal heating and vapourization of water
Autoclaving	110 - 130	10 - 15	steam	MTMT - raw material heated with water under pressure in a sealed vessel
Toasting 100 kPa	90 - 105	30 - 45	steam	LTLT - raw material steamed at atmospheric pressure
Toasting 400 kPa	100 140	1 - 10	steam	HTST - steam added to treatment vessel under pressure
Dry roasting	90 - 100	0.2 - 2	gas/ electicity	HTST - heat transfered by conduction to raw material dispersed in a hot granular medium
Flaking	90 - 95	10 - 20	steam	LTMT - raw material steamed in a tempering vessel then rolled

HTST - high temperature/ short time LTLT - low temperature long time MTMT - medium temperature medium time Source: Melcion and van der Poel (1993)

The data in Table 1.3.9. show a good degree of consensus on the apparent ileal digestibility of N in solvent extracted defatted soyabean meal, which was the most frequently investigated product. This appears to be superior to any of the full fat processed soyabean meals, although it is difficult to draw definitive conclusions as there is little data available on the ileal N digestibility of full fat products. Ethanol extracted soya protein concentrate and isolated soya protein appear to be more digestible than the other products. This supports an observation by Lalles *et al.* (1993) that extra processing steps such as ethanol extraction appear to reduce antigenicity of soya protein in calves and piglets. Hancock *et al.* (1990) observed 4.9 and 3.9 fold improvements in ADG and FCR respectively in pigs fed diets containing ethanol extracted raw soya flakes compared to untreated raw soya flakes. A further 2.5 fold increase in ADG and 1.5 fold increase in FCR occurred when pigs were fed ethanol extracted autoclaved soya flour.

There is no doubt that processing increases utilization of soyabean protein by pigs and it would appear from the limited data reviewed here that extra processing steps are of benefit particularly for young pigs. It is possible that enzyme treatments that partially hydrolyse soya protein could reduce the need for extra processing. Evidence to support this comes from the long tradition of fermenting cooked soyabeans to provide a range of soya bean foods for humans (Lin 1991).

Table 1.3.9. Apparent ileal digestibilities of N in some commercially processed soyabean products

Soyabean/ process	Apparent ileal N digestibility (%)	Reference
DF SBM	78.9	Vandergrift 1983
DF SBM (44% CP)	77.3	Rudolph *et al.* 1983
DF SBM (48% CP)	79.6	
DF Soy flour	78.9	
FFS extruded	78.1	
SBM (44% CP)	79.5	Walker *et al.* 1986
soya protein concentrate (eth. extr.)	89.7	
Isolated soya protein	90.6	
DF SBM	78.0	Knabe *et al.* 1989
FFS extruded	75.0	
Isolated soya protein	88.32	Sohn *et al.* 1994
Soya protein concentrate (eth. extr.)	87.65	
DF SBM (48.5% CP)	77.29	
FFS extruded	75.6	Marty *et al.* 1994
FFS jetsploded	69.0	
FFS micronised	71.7	
FFS roasted	69.5	
DF SBM	81.6	
soyabean concentrate	86.8	Grala *et al.* 1998
FFS dehulled roasted	82.8	

DF SBM defatted (solvent extracted) soyabean meal FFS full fat soyabean
CP crude protein

Table 1.3.10. Effect of specific heat treatments on apparent ileal digestibility of N in raw soya bean .

Heat treatment	Apparent ileal N digestibility %	Reference
FFS steamed 110°C for 20 min	76.4	Herkelman *et al.* 1992
FFS steamed 102°C for 10 min.	51.1	Qin *et al.* 1996
FFS steamed 102°C for 20 min	68.3	
FFS steamed 102°C for 40 min	81.2	
FFS steamed 120°C for 2 min	70.3	
FFS steamed 120°C for 7.5 min	82.0	
FFS steamed 134°C for 1.5 min	80.2	
RSF autoclaved 120°C 15min	77.1	Li *et al.* 1998

FFS full fat soyabean RSF defatted soya flakes

1.4. Enzyme applications for soyabean protein

1.4.1 *Protease treatment of soyabeans.*

Traditionally, fermentation of soyabeans has been used to improve functional properties of soyabeans such as texture, flavour and keeping qualities as well as the nutritional value. Fermented soya foods employ microbial enzymes via the fermentation process to hydrolyse soya proteins to varying degrees prior to consumption. Fermentation by *Rhizopus oligosporus* to produce tempeh improves the apparent protein digestibility in rats as does fermentation by *Bacillus natto* to produce natto (Lin 1991).

Zamora and Veum (1979) fed heated soyabeans fermented with either *Aspergillus oryzae* or *Rhizopus oligosporus* to growing pigs. Pigs fed diets containing beans fermented with *A. oryzae, R. oligosporus* and unfermented beans had average daily gains of 0.60, 0.56 and 0.52 kg and feed conversion of 2.04, 1.96 and 2.33 respectively. Respective net N utilization in these pigs was 55.7%, 55% and 50.6% indicating that improvements in performance were due to improved availability of amino acids in the fermented beans.

Ikeda *et al.* (1995) demonstrated differences in *in vitro* protein digestibility in a number of Japanese soya foods. Protein digestibilities of 92% (whole boiled soyabean), 100% (protein lipid film), 93% (soyabean curd), 90% (fermented whole beans - natto) and 78% (roasted soyabean meal) were observed. Electrophoretic analysis and peptic digestion studies revealed differences in the chemical form of the proteins in these foods and led the authors to conclude that the chemical form of soya protein imparted by processing or fermentation is an important factor in digestibility. The scope for improving the functional and digestive properties of soya protein through the use of refined microbial enzymes has been appreciated for many years and much of the research on the enzymatic modification of soya protein has focused on the improvement of the functional properties, e.g. gelation

and flavour, of isolated soya protein for use in the human food industry (Arai and Fujimaki 1991).

Some early work on the addition of proteases to pig diets was aimed at supplementing digestive enzyme insufficiencies in the young pig. Lewis, Catron, Lui, Speer and Ashton (1955) conducted a series of five studies in which diets for early weaned pigs (6 - 10 days) containing purified soyabean protein were supplemented with pancreatin and /or pepsin, papain, a diastatic protease (from *A. oryzae*) or a fungal protease (Star-Zyme P). They reported improvements in FCR of 0.62, 0.64, 0.46, and 0.51 for diets supplemented with pepsin, pepsin + pancreatin, papain and diastatic protease respectively compared with control soya protein diets. However, pigs fed enzyme supplemented soya protein diets performed less well than pigs fed milk-based diets.

The serine protease inhibitors in raw soyabean appear to be a major contributor to its poor digestibility. As these are proteins it is feasible to suggest that they could be inactivated by enzymatic degradation. However, as Classen *et al.* (1993) point out the structural integrity of the active site of the serine protease inhibitors, KTI and BBI, conferred by disulphide bridges is likely to be resistant to proteolysis. This viewpoint is supported by Vaintraub and Haram (1995) in studies on the influence of proteolysis on the activity of KTI. Reduction in inhibitory activity was only achieved when KTI was degraded to TCA soluble peptides when digested with either papain, substilisin or pepsin. Initial work into the effect of a range of microbial enzymes on the activity of serine protease inhibitors has been encouraging. Meijer and Spekking (1993) isolated micro-organisms that would utilize KTI and BBI as sole carbon, nitrogen and sulphur sources and inactivate them over a time course of several days. Nine strains of fungi and seven strains of bacteria that could catabolize and inactivate KTI and BBI were isolated. These micro-organisms must all

possess enzymes capable of hydrolysing the serine protease inhibitors in soyabeans. However, this does not necessarily mean that SPI's would be utilized in preference to the other sources of C, N or S in whole soyabeans. Further work by Huo, Fowler, Inborr and Bedford (1993) identified several microbial enzymes capable of reducing the activity of KTI and lectins in raw soyabean and low temperature (105° C) extruded soyabean (LTES). An acid fungal protease, a neutral bacterial protease and three alkaline bacterial proteases were investigated. The most efficacious of these proved to be two alkaline bacterial proteases with pH optima of 10 and 8 which reduced trypsin inhibitor levels in RSB and LTES by 96% after 12 hours incubation at an inclusion level of 1%. However, there is no published data available on the efficacy of protease-treated raw soyabean included in pig diets.

Few studies have been undertaken examining the effect of pretreatment of processed soyabean meals with exogenous proteases on the growth performance of pigs. Leibholz (1981) fed early weaned pigs (7 days) with diets containing isolated soyabean protein partially hydrolysed by papain. No significant differences were observed in average daily gain, feed conversion ratio, N retention or N digestibility over a 3 week feeding period when compared with diets containing unhydroylsed isolated soya protein.

Nasi (1991) investigated the efficacy of different methods of application of a multi-enzyme mix (cellulase, protease and β-glucanase) to solvent extracted SBM for inclusion into diets of growing pigs. The enzyme application regimes and results of this study are summarized in Table 1.4.1.

Table 1.4.1. Summary of crude protein digestibility and nitrogen retention of diets containing enzymically treated SBM in growing pigs (30 - 75 kg).

Enzyme application regime	Crude protein digestibility	Nitrogen retention (of intake)
no enzyme treatment	0.817	0.425
0.1% enzyme mix added to dry SBM	0.865	0.423
0.1% enzyme mix added to wet SBM* 8h prior to feeding	0.879	0.430
0.1% enzyme mix sprayed onto conditioned (60°C, 30% moisture) SBM 2h prior to feeding	0.887	0.448

* moisture content not stated
Adapted from Nasi (1991)

Although the results showed a trend towards increased utilization with enzyme treatments the differences were not significant. However, it is worth noting that the best results were obtained (with the latter two treatments in Table 1.4.1.) when the multi-enzyme mix was allowed time to act prior to feeding. In a subsequent growth performance trial Nasi (1991) observed no significant differences in ADFI, ADG, or FCR between pigs fed diets containing conventional SBM, extruded SBM or enzyme pre-treated extruded SBM. Spring, Wenk, Lemme, Bee and Gebert (1998) investigated the effect of Vegpro (Alltech inc.), a multi-enzyme complex containing protease (main activity), cellulase, pentosanase, α-galactosidase and amylase, topically applied to grain/soya diets at 3 different lysine concentrations, on the performance of 4 week old newly weaned pigs. They observed no significant differences in ADG. However, feed conversion ratios were improved by 3.5%, 1.75% and 8.8% when pigs were fed diets with lysine concentrations of 1.20%, 1.08% and 0.97% respectively. Because multi-enzyme mixtures were used in these two studies it is not possible to attribute any improvement in pig performance to the action of any single enzyme. Although the improvements in crude protein digestibility and N retention obtained by Nasi (1991) suggest an increase in availability of amino acids this may not necessarily have been due to protease action, as NSP hydrolysing enzymes may degrade

plant cell walls and make substrates for digestive enzymes more accessible (Bedford and Schulze 1998).

Hessing, van Laarhoven, Rooke and Morgan (1996) and Rooke, Slessor, Fraser and Thomson (1998) compared the effect of pretreating defatted SBM (48% crude protein) with an alkaline protease (P1) from a *Bacillus* species with an acid protease (P2) from an *Aspergillus* species. Pretreatment consisted of incubating a SBM slurry (20% dry matter) with 1mg g^{-1} SBM (dry matter) at 50° C for 3 hours at the appropriate pH. *In vitro* investigations showed antigenicity of soya protein was reduced by 26% and 6% after treatment with P1 and P2 respectively (Hessing *et al.* 1996; Rooke *et al.* 1998). Significant hydrolysis of glycinin and β-conglycinin in raw soyabean was observed after pretreatment with P1 (Hessing *et al.* 1996). However, in contrast to these results when diets containing the protease treated SBM's were fed to 28 day old newly weaned pigs (Hessing *et al.* 1996; Rooke *et al.* 1998) or 7 day old chicks (Hessing *et al.* 1996) treatment with P2 improved growth performance whereas P1 had no effect. Although the overall improvement in growth performance of the piglets fed P2 treated SBM was not significant, there were significant increases in ADG from 0.064 to 0.120 kg day^{-1} for the first 7 days. Rooke (1998) made a similar observation in a subsequent feeding trial involving 24 piglets per diet, the P2 treatment increasing ADG from 0.121 to 0.155 kg day^{-1} for the first 7 days. The overall results of these trials are summarized in Table 1.4.2. The lack of response to treatment with P1 may possibly be due to the formation of lysinoalanine in the soya protein treated by both enzyme hydrolysis and alkali. Lysinoalanine is formed by the hydration of cystine and its subsequent reaction with lysine residues under alkaline conditions. Lysinoalanine reduces the nutritional value of protein due to destruction of cystine, cysteine and lysine (Pearson 1983), and its production depends on temperature, exposure time and type of protein. In these studies the treatment of soya protein with alkali alone did

not result in poor animal performance, therefore it was unlikely that significant amounts of lysinoalanine were formed. However, it is possible that hydrolysis by P1 may have exposed vulnerable amino acid residues to the alkali and resulted in lysinoalanine production and subsequent reduced performance.

Caine, Sauer, Tamminga, Verstegen and Schulze (1997b) pre-treated SBM (48% crude protein) with subtilisin from *Bacillus subtilis,* and investigated the effect of its inclusion into diets on the ileal digestibility of amino acids in 4 week old newly weaned pigs. Protease pretreatment consisted of either spraying the enzyme onto SBM at an inclusion rate of 1μl g^{-1} SBM or incubating SBM (1:2 wt/vol. in distilled water pH adjusted to 4.5) with the same inclusion rate of protease at 50° C for 16h. No significant differences were observed in ADFI, ADG or FCR between the protease-treated meals and their appropriate controls, nor were there any significant differences in the apparent ileal digestibility of amino acids in diets treated with protease compared to untreated diets. The performance data from this trial are included in Table 1.4.2.

Table 1.4.2. Summary of the results of feeding protease treated soyabean meal to pigs or chicks.

age of pigs (days)	Initial body wt (kg)	duration of trial days	Enzyme treatment	ADG		FCR		Reference
				+ protease	- protease	+ protease	- protease	
30	7.57	14	P1	0.153	0.199	1.74	1.39	Hessing *et al.* 1996
			P2	0.181	0.128	1.54	1.86	
-	-	21	P2	0.271	0.257	1.45	1.52	
21	6.4	9	subtilisin - sprayed	0.104	0.160	3.26	2.35	Caine *et al.* 1997
			subtilisin - incubated	0.148	0.152	2.32	2.25	
29	7.6	14	P1	0.153	0.199	1.71	1.59	Rooke *et al.* 1998
			P2	0.181	0.128	1.54	1.83	
29	7.3	21[1]	P2	0.271	0.257	1.47	1.56	
-	8.5	7	P2	0.135	0.119	2.02	2.10	
chicks								
7	0.141	27	P1	0.034	0.034	1.125	1.97	Hessing *et al.* 1996
			P2	0.041	0.030	2.13	1.93	

[1] Production trial

P1 and P2 diets - treatment consisted of incubation of SBM (20% DM) with 1mg g^{-1} SBM (DM) of P1 or P2 at 50°C for 3 h at pH 8.5 (P1) or pH 4.5 (P2)
control diets (- protease) were treated as above without the protease

substilin treated diets : sprayed - SBM was sprayed with 1µl g^{-1} SBM of subtilisin
incubated - SBM (33% DM) was incubated with 1µl g^{-1} SBM (DM) of subtilisin at 50°C for 16h at pH 4.5
control : treated as above without subtilisin

1.4.2. *In vivo Evaluation of protease treatments.*

The aim of supplementing a pig diet with proteases is to increase the utilization of protein in that diet which will ultimately be reflected in an increase in lean tissue accretion. There is a limit to the pig's capacity for lean tissue accretion which depends on a number of factors including stage of development, sex, genotype and adequacy of nutrition (English *et al.* 1996). In the case of protein provision the achievement of optimum growth and lean tissue deposition rates depends, not only on the amount of protein supplied, but also on the protein quality, its digestibility and an adequate supply of energy to enable the pig to fully utilize the protein provided (Whittemore 1993; English *et al.* 1996). The dependence of protein utilization on energy supplied by the diet has led to the practice of expressing protein requirements in terms of a ratio of protein to digestible energy, expressed as g lysine MJ DE^{-1} as lysine is the first limiting amino acid in most diets (English *et al.* 1996). Well formulated diets providing the correct lysine:energy ratio and sufficient in all other nutrients for a pig of a particular age, sex and genotype will result in optimum growth and lean tissue deposition rates. If the pig is already growing at its maximum protein deposition rate any increase in protein availability or digestibility will not result in detectable increases in utilization as protein in excess of requirements will be deaminated.

Therefore, in order to evaluate the effectiveness of protease treatments in increasing dietary amino acid availability it is necessary to reduce the lysine:energy ratio of the diet below the level required for maximum protein deposition by that age/weight/genotype of pig. If the enzyme treatment is effective then the pig should respond with increased growth rate and lean tissue deposition, when compared to control pigs on identically formulated diets. The study of Spring *et al.* (1998) outlined in section 1.4.1. provides evidence to confirm this. As dietary lysine concentrations of the diets were decreased from 1.20% to 1.08% and 0.97%, FCR of the pigs fed Vegpro (Alltech inc.) supplemented diets improved by 3.5%,

1.75% and 8.8% respectively when compared with the appropriate untreated diets. Indeed, if lysine:energy ratios are not adjusted in this way effective enzyme treatments may not only go undetected but may also appear to be detrimental. The latter situation may arise because amino acids absorbed excess to requirements are deaminated with the nitrogenous products, being excreted as urea and ammonia via the kidneys. The processes of deamination and excretion require both energy and water. In addition high blood plasma levels of some amino acids, notably lysine, tryptophan and methionine, can exert toxic effects which can reduce performance (Cole 1985; English *et al.* 1996).

The energy costs of deamination and excretion are 5 MJ and 7MJ kg^{-1} protein deaminated respectively (Whittemore 1993). Although the carbohydrate residue of amino acids after deamination provides approximately 12 MJ kg^{-1} protein deaminated (Whittemore 1993), this represents inefficient use of resources as the pig is using the extra protein as an energy source. Each MJ of energy provided by protein requires approximately 840g of water to metabolize (Brooks and Carpenter 1990). The pig's demand for water has been shown to increase dramatically once the protein requirement for lean tissue accretion is exceeded (Pfeiffer and Henkel 1991; Brooks 1994). The resultant increase in water intake required to meet this demand may contribute to the total volumetric fill of the pig at the expense of feed intake (Brooks 1994).

It is not clear from the latter 3 papers reviewed in the previous section whether the subtilisin used by Caine *et al.* (1997b) and the acid protease (P2) used by Hessing *et al.* (1996) and Rooke *et al.* (1998) are the same enzyme. However, the pretreatment regimes were remarkably similar and both enzymes were supplied by Finnfeeds International Ltd. The lack of response to enzyme inclusion in the study conducted by Caine *et al.* (1997b) can be explained by the apparent failure of these authors to realize the implications of

dietary protein:energy ratios. The studies conducted by Hessing *et al.* (1996) and Rooke *et al.* (1998) had dietary lysine:energy ratios ranging from 0.82 to 0.86 g lysine MJ DE^{-1}. The recommended lysine:energy ratio for pigs of this weight (5 - 10 kg) varies from 0.95 g lysine MJ DE^{-1} (Whittemore 1993; English *et al.* 1996) to 0.85 g lysine MJ DE^{-1} (calculated from NRC (1988)) and 0.81 g lysine MJ DE^{-1} (calculated from NRC (1998)). These figures have generally been calculated from compilations of performance data and the differences between them reflect both difference between pig genotype and feed components generally used in the pig industries in Europe and North America at the time of compilation (Knabe 1996). The studies of both Hessing *et al.* (1996) and Rooke *et al.* (1998) were conducted in the UK, using barley and maize based diets and although the genotype of pig was not stated it was presumably of a typical UK genotype. Based on these criteria their diets were formulated to contain a sub-optimal lysine:energy ratio which is reflected in the trend towards increased performance observed with protease treated SBM. On the other hand, Caine *et al.* (1997b) used a cornstarch based diet and state that their diets were formulated to include the essential amino acids at levels of 0.1% to 0.8% above NRC (1988) standards for that age of pig (genotype not stated). This could explain why they did not obtain a similar response to the same enzyme treatment.

There is also evidence to suggest that amino acid absorption across the ileum reaches a maximum rate. Fan, Sauer, Hardin and Lien (1994) determined apparent ileal digestibilities of sixteen amino acids in six diets ranging from 4% to 24% crude protein in growing pigs. For all amino acids tested there existed threshold inclusion levels above which apparent ileal digestibility did not increase. The NRC (1988) standards for the age of pig used by Caine *et al.* (1997b) and hence the amino acid concentrations in their diets, are above the threshold levels reported by Fan *et al.* (1994), a fact which Caine *et al.* (1997b) acknowledge in the discussion section of their paper. The results presented by Fan

et al. (1994) suggest another physiological mechanism whereby the pig fails to respond to increasing dietary amino acid concentrations, namely, that amino acid transport systems across the ileal epithelia become saturated, resulting in a failure to absorb excess amino acids whether they be of exogenous or endogenous origin.

The above points highlight an important concept in the experimental design of feeding trials, namely, the need to ensure that the pig is physiologically able to respond to dietary treatments in a manner that will answer the question being asked of it.

1.5. Rationale and objectives.

The use of exogenous proteases to target the protein component of pig diets needs a different approach to that of the non-starch polysaccharride degrading enzymes. Two factors favour the use of NSP degrading enzymes in monogastric animal diets and allow them to be employed successfully in whole compound feeds. Firstly, they target the main ingredient of the diet, the cereal component, which often represents 75 % of the whole diet. The NSP content of cereals is between 10% and 15 % (Charlton 1996). Therefore, whilst the substrate for these enzymes represents only a fraction of the total feed, the NSP degrading enzymes are highly specific and are not subject to competition from different substrates. Secondly, they supplement the animals' digestive enzymes with enzymes it does not itself produce, therefore as long as they remain active in the digestive tract they contribute to the degradation of substrates that the animal cannot utilize. These factors do not hold true when targeting the protein supplements in whole diets with proteases. The vegetable protein meals used as protein supplements in compound feeds represent only a fraction of the total feed, typically 20 % - 30 %. The target substrate for the enzyme will be even less than this. For example if full fat soyabean meal with a crude protein content of 40 % is included in a diet at a rate of 25 %, then the actual soyabean protein content of

that compound feed will be 10 %, and the ANF content even less, at 6-7 % of the total protein (Nielsen 1983). This represents a considerable dilution of the substrate by other feed components. Also proteases will be subject to competition between different proteins in a complete diet. If proteinaceous ANF's are percieved as the target substrate, the competition from other feed proteins may result in little or no ANF degradation. Animals also synthesize their own proteases, which digest protein with varying degrees of efficiency depending on its source. Data for apparent ileal protein digestibility from various studies indicate that the protein in full fat soyabean meal is less digestible than that in solvent extracted soyabean meal (Table 1.3.9.). Therefore, these products present an opportunity for improvement by exogenous enzyme applications. Raw soyabean has a low nutritional value due to proteinaceous ANF's and also presents a promising substrate for improvement by the application of exogenous proteases. However, there is probably little to be gained by simply adding proteases to compound diets. On the other hand, if proteases are used to pretreat protein components of the diet and present the animal with a partially hydrolysed product, this may increase protein digestibility by altering the starting point of endogenous proteolysis.

Pretreatment of soyabean meals with exogenous enzymes prior to inclusion into the complete diet not only overcomes the dilution effect of other feed ingredients, but also allows the effect of enzyme action to be assessed prior to feeding. This is a pertinent point when considering the addition of exogenous proteases in animal feeds, as it is impossible to detect exogenous protease activity *in vivo*. Although immunochemical techniques such as ELISA's may be employed to detect the presence of a protease these give no indication of functionality and substrate degradation or end product production cannot be measured *in vivo* as it is impossible to separate the effects of exogenous and endogenous protease activity. If the goal of exogenous protease pretreatment is to present the pig with a more

digestible protein, liquid feed systems present a cost effective way to achieve it. Individual dietary components can be pretreated in an aqueous environment prior to incorporation into the whole diet. However, the conditions under which enzyme addition to liquid feed systems will be employed needs to be borne in mind when developing pretreatment regimes, to enable the technology to be successfully transferred from a research environment to a commercial one. In terms of cost effectiveness alone this means developing application conditions that do not require high energy inputs.

Studies on the use of proteases to improve the utilization in pig feeds have largely focused on the use of solvent extracted soyabean meals in newly weaned pig diets. There is no doubt that the newly weaned pig presents a challenge in this area as its immature digestive system does not allow it to utilize vegetable proteins well (English *et al.* 1996). However, due to the almost ubiquitous inclusion of soyabean meals into grower and finisher pig diets even small increases in utilization could have cost benefits. No data have been published to date on the effect of protease pretreatment of full fat SBM or RSB on the utilization of these products by grower or finisher pigs.

The objectives of this study were to:

- assess the efficacy of three microbial proteases in:
  - partially hydrolysing soyabean protein.
  - reducing levels of trypsin inhibitors in raw soyabean.
- establish conditions needed to achieve partial hydrolysis of soya protein which could be undertaken on the farm.
- assess the effect of protease treated raw soyabean and processed soyabean meal on the growth performance of grower and finisher pigs.

Chapter 2.

The determination of a pretreatment strategy for raw soyabean and evaluation of methods for determining protein hydrolysis in steeped raw soyabean pretreated with proteases.

2.1 General introduction.

The application of exogenous proteases to raw soyabean to be used in liquid feed systems for pigs allows pretreatment of the substrate prior to incorporation into the diet. However, there is little point to pretreatment if it does not result in some degree of degradation of the substrate. Pretreatment regimes need to be developed in order to elicit the desired effect on the substrate. As it is envisaged that enzyme pretreatment will eventually take place under conditions prevailing on farm, it was felt necessary to impose a temperature constraint on the regime to be developed. Therefore all pretreatments were conducted at 20° C. Caine *et al* (1997a) determined the optimum conditions for *B. subtilis* subtilisin pretreatment of SBM by measuring increases in protein solubility. They found the optimum conditions to be incubation at 50°C and pH 4.5. However, it would be difficult to justify the energy cost of the pretreatment conditions used by Caine *et al.* (1997a) in an on farm process.

The exogenous enzymes investigated in this study were all microbial proteases supplied by Finnfeeds International Ltd (Marlborough UK). The source organism and specificity were unknown; however information was available on the activity and pH optima of the proteases. As proteases they could fall into one of two categories, endopeptidases or exopeptidases. Endopeptidases cleave peptide bonds within protein molecules whereas exopeptidases attack terminal peptide bonds, either at the amino terminal end of a peptide (aminopeptidases) or at the carboxyl terminal (carboxypeptidases), and liberate free amino

acids. Endopeptidases split proteins into progressively smaller polypeptides. They generally exhibit a degree of specificity in as much as they will cleave peptide bonds between specific groups of amino acid residues. For example, the digestive enzyme trypsin, an endopeptidase, cleaves peptide bonds in which the carboxyl group is supplied by either lysine or arginine, whereas chymotrypsin primarily cleaves bonds in which the carboxyl group is supplied by phenylalanine, tryptophan or tyrosine but will also cleave those supplied by asparagine, histidine and methionine albeit at a slower rate (Voet and Voet 1995). Therefore, each protein molecule presents a number of substrates which will, in effect, compete for the active site of an endopeptidase. The structure of the protein may also affect the availability of scissile bonds. In globular proteins the scissile bonds specific to a particular enzyme may be protected within the globular structure and be largely inaccessible. The main storage proteins of legume seeds are globular in structure and this may contribute in part to the inherent indigestibility of native legume proteins compared with denatured legume proteins. Guo *et al.* (1996) found that native soyabean protein was less susceptible to hydrolysis by a neutral protease from *B. subtilis* than heat denatured soyabean protein. Some of the antinutritional factors in raw soyabean are themselves proteins and these present attractive targets for protease treatment. However, it should be remembered that they only represent a small proportion of the total protein complement. In soyabeans the serine protease inhibitors (SPI), the Kunitz trypsin inhibitor (KTI) and Bowman-Burke trypsin/chymotrypsin inhibitor (BBI) constitute approximately 6% of the total protein (Hymowitz 1984). In terms of substrate concentration, i.e. the number of scissile bonds they present for any given protease, their concentration is small compared to that of the storage proteins, glycinin and β-conglycinin, which contribute approximately 90% of the total protein. Therefore, the main effect of protease pretreatment is likely to be on the storage proteins.

A number of methods have been used to assess the efficacy of protease pretreatments of soyabean meals. Rooke *et al.* (1996) aimed to assess whether protease treatment of SBM could reduce its *in vitro* antigenicity and improve its nutritional value when fed to newly weaned piglets. Antigenic soya proteins were measured by a competitive Enzyme Linked Immunosorbent Assay (ELISA) technique. The SBM treated with protease contained less antigenic proteins than the other diets containing soya. It was assumed here, as in many studies, that reduction in levels of antigenic proteins as measured by ELISA, indicates protein denaturation by exogenous protease treatment. This is not necessarily an accurate assumption as for example, antiserum from animals immunised with soyabean protein may not recognise any protein components of an enzyme treated soya product, but the treated product may still contain antigenic epitopes (J. Thorpe, University of Bristol, personal communication). Hessing *et al.* (1996) examined the ability of two microbial proteases (an alkaline protease, P1 and an acid protease, P2) to degrade ANFs, and to determine whether enzymatically hydrolysed SBM could improve the biological performance of newly weaned piglets or broiler chicks. The SBM was pretreated with the protease before feeding. Sodium dodecyl-suphate polyacrylamide gel electrophoresis (SDS PAGE) and Western Blotting analysis demonstrated that P1 could significantly hydrolyse the storage proteins glycinin and β-conglycinin, and to a certain extent KTI, at inclusion levels of 1000 - 10000 units g^{-1} material, but there were no effects on soya bean lectin. It was cautioned that *in vitro* immunochemical analysis of ANFs must be interpreted with care as the hydrolysis of proteins such as trypsin inhibitors may expose more antigenic epitopes but the inhibitor itself may not be functional, resulting in misleading results. Rooke *et al.* (1998) used SDS-PAGE and a soluble α-amino nitrogen (α-AN) assay to examine the effects of two proteases (an alkaline protease, P1 and an acid protease, P2) on SBM. Pretreatment changed the composition of SBM, and soluble α-AN concentrations were increased by treatment with P1 and P2. P1 reduced β-conglycinin concentration (as

determined by SDS-PAGE) to a greater extent than P2. Walsh, Headon and Power (1997) also measured increases in soluble α-AN to assess the effect of protease treatment of rapeseed meal. They demonstrated a 54 % increase in soluble α-AN compared to the control sample as a result of protease treatment (cited by Pluske and Lindemann (1998)).

These studies indicate that a number of methods are available to determine the effect of protease pretreatment of soyabean protein. Immunochemical techniques such as ELISA appear to be of limited value in this respect as the results are difficult to interpret in terms of protein degradation. The production of α-AN appears to be a useful measurement as it gives an indication that protein hydrolysis has occurred but elicits no information on the degradation of individual proteins, whereas electrophoresis may yield information about the patterns of hydrolysis of different proteases.

In the following series of studies the use of a technique to determine α-AN was evaluated as a means of measuring hydrolysis of soyabean protein. SDS PAGE was also evaluated as a means of determining the hydrolysis patterns of the proteases. Although it would be desirable to determine a range of ANF contents of protease treated raw soyabean, the equipment necessary to effectively determine lectin content was not available, therefore ANF determination was limited to that of the trypsin inhibitors.

2.2 Assessment of the viability of using the A.O.A.C. (1992) method for the determination of free amino nitrogen in wort to detect α-amino nitrogen in soyabean slurry.

2.2.1 *Introduction.*

The A.O.A.C. (1992) method for the determination of free amino nitrogen in wort uses ninhydrin to detect α- amino groups of amino acids. Ninhydrin forms a purple complex with the nitrogen atom of α-amino groups present in free amino acids and amino groups at the N-terminal end of proteins and peptides. The intensity of colour is proportional to the amount of α-amino nitrogen present (Voet and Voet 1995) and can be quantified by spectrophotometry. Therefore, it should be possible to detect increases in terminal amino groups due to protein hydrolysis using this method. As it is a spectrophotometric method it is necessary for the reactants to be in solution. Fortunately, soyabean protein is readily soluble in water above pH 6.3 (Pearson 1983), enabling the protein fraction to be extracted with ease.

The objective of this study was to determine if α-AN could be detected in ground soyabean slurries using the A.O.A.C. (1992) method.

2.2.2 *Materials and Methods.*

Raw whole soyabeans were ground in a hammer mill through a 1.5mm screen. Triplicate samples of 50g ground raw soyabean were mixed with 250ml tap water, and steeped at room temperature (19°C ± 4°C) for 3 days. Subsamples of 1ml were taken daily using a wide-mouthed pipette. Subsamples were transferred to centrifuge tubes and 5ml distilled water added. The samples were mixed with a vortex mixer for 1min and centrifuged at

10 000 r.p.m for 10 min. The resulting supernatant was diluted 1 in 40 , to give a final sample dilution of 1 in 200. This was used for the α-AN assay which was conducted according to the procedure of A.O.A.C. (1992). Three replicate analyses of each of the three samples were conducted daily giving a total of 9 readings per day.

2.2.3 *Results.*

The α-AN levels detected in the raw soyabean slurry increased with time over the 3 days from 0.77 mg α-AN g^{-1}soya to 2.14 mg α-AN g^{-1}soya. The results are presented in Figure 2.2.1.. The A.O.A.C. (1992) method calculated α-AN levels in mg l^{-1}, and while this was a relevant measure for wort it was felt that it was not appropriate for soyabean, therefore, in this instance α-AN was expressed in mg g^{-1} soyabean.

$$\alpha\text{-AN mg } g^{-1} = (A_s - A_b) / (A_{std} - A_b) \text{ X } 0.002 \text{ X } D/S \qquad \text{equation 1}$$

where A_s, A_{std} and A_b = absorbance of sample, standard and reagent blank respectively, D = dilution factor, S = g soya in sample and the standard contains 0.002 mg α-AN ml^{-1}.

The error bars on Figure 2.2.1 show the standard deviation and indicate the scatter of the data obtained from the 9 replicates. Standard deviations of 0.106, 0.243, 0.196 and 0.257 and coefficients of variation of 13.8%, 19.4%, 9.7% and 12.0% were calculated for days 0 - 3 respectively.

2.2.4. *Discussion.*

The increase in α-AN observed over the 3 day experimental period is likely to be due to the action of endogenous proteases in the raw soyabean. The normal physiological function of these proteases is to degrade storage proteins into a source of nitrogen to be used by the developing seedling during germination (Spencer 1983).

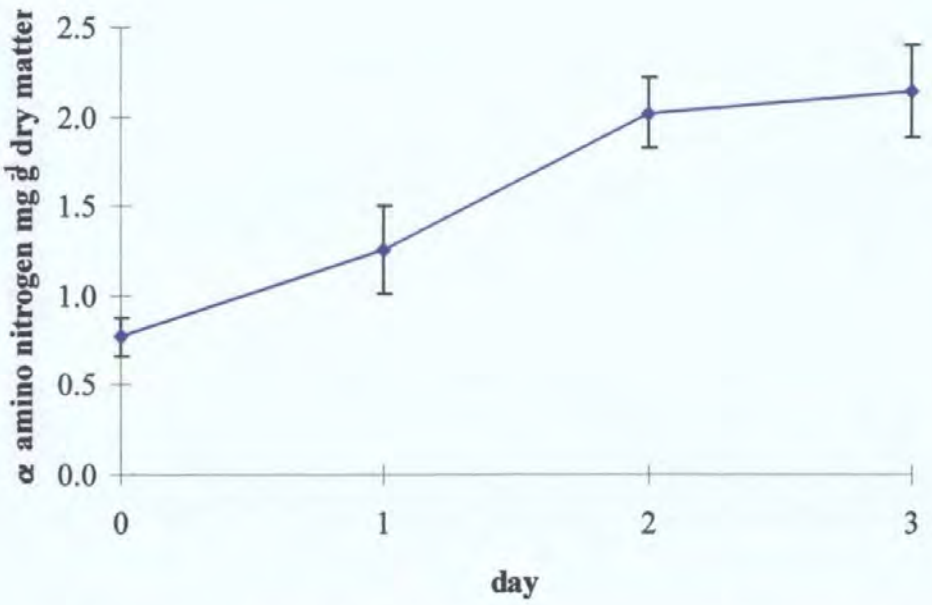


Figure 2.2.1 Changes in α- Amino Nitrogen detected in raw soyabean slurry steeped for 3 days at 19 ± 4° C. Error bars indicate one standard deviation.

Table 2.2.1 Average absorbance of 3 replicates and coefficient of variation (CV) within replicates for α- amino nitrogen (mg g^{-1} DM) in the standard and soyabean slurry samples.

		Time (days)				total CV %
sample		0	1	2	3	over 3 days
Standard	av.	0.45	0.47	0.47	0.47	
	CV	2.22	2.44	3.19	3.43	3.19
Sample 1	av.	0.18	0.26	0.47	0.52	
	CV %	3.15	7.33	1.23	3.47	
Sample 2	av.	0.19	0.31	0.44	0.43	
	CV %	5.26	8.21	0	6.15	
Sample 3	av.	0.22	0.37	0.52	0.55	
	CV %	13.32	10.33	10.66	4.20	

The data presented in Figure 2.2.1. show that there was a considerable degree of variation between samples. It was felt that this variation was due to two contributory factors. Firstly, it is likely that particle size may affect extractable α-AN. Larger particle sizes expose less surface area for protein to be extracted, and more cells will remain intact which will also reduce the amount of extractable protein. It was difficult to obtain an homogenous subsample of the soya slurries due to the variation in particle size in the ground samples. Even though slurries were stirred vigorously immediately prior to sampling, the larger particle sizes settled very quickly. This meant that subsamples did not necessarily reflect the distribution of particle sizes in the whole sample and the proportions of particle sizes between subsamples differed. Secondly, in calculating the α-AN content in mg g^{-1}soya it was assumed that all soluble α-AN from each subsample was extracted into the 5ml distilled water added prior to centrifugation. However, in this study no other extraction procedures were tested and it is possible that additional steps may have been necessary to extract the maximum amount of soluble protein. Therefore, in retrospect the amount of soluble protein extracted may have varied between samples. Again, this may have been due to differences in the relative proportions of different particle sizes between the samples.

Of more concern was the fact that there were marked differences in α-AN levels between replicates of the same sample. The means and coefficient of variation between absorbance readings of replicates of samples taken on each day and of the glycine standard are presented in Table 2.2.1. This variation reflected difficulties encountered with the method itself which may have affected the reliability. The method required the reactant tubes to be placed in a boiling water bath for exactly 16 min for ninhydrin to react with α-AN. In the absence of an adequately controlled boiling water bath this was done by placing test tubes in batches of 15 (3 x 3 sample, 3 standard, 3 reagent blank) in a pan of boiling water over a

burner. Not only did this make the temperature of the bath difficult to control, but exact timing of removal of each tube was also more difficult as it took approximately 1 min to remove all 15 tubes. The relatively small variability between the standard replicates was probably due to these tubes being removed first.

This experiment showed that it was possible to use the A.O.A.C. (1992) method for the determination of free amino nitrogen in wort to detect α-AN in soya slurries. However, it was concluded that the method needed to be refined in the following ways.

- Determination of the effect of particle size on the extraction of α-AN.
- Standardization of the extraction of the soluble protein fraction to ensure the maximum amount of protein is extracted.
- Modification of the method to increase reliability.

2.3 Development of an extraction procedure for the determination of α-amino nitrogen in soyabean slurry.

2.3.1. *Introduction*

The study discussed in section 2.2 highlighted the need to establish an extraction procedure for soluble α-AN in soyabean slurry. Wolf (1978) recommended water, dilute alkali (pH 7 - 9) or 0.5 - 2 M sodium chloride as extraction solvents for soyabean protein in order to obtain protein in an undenatured state. Water was chosen as the solvent in this case as the effect of the latter two solvents on the α-AN assay was unknown. Wolf (1978) also suggested that a 1:10 meal : solvent ratio yielded an adequate amount of protein. However, for the purpose of this assay the total soluble protein needed to be extracted. In the previous experiment (2.2) a soya : solvent ratio of 1:20 was used. The objective of this experiment was to determine if a soya : solvent ratio of 1:20 was sufficient for the extraction or the total soluble protein or if further extraction was necessary.

2.3.2 *Materials and methods.*

The experiment was designed as a two factor factorial design in which the first factor was the number of extraction steps, 1, 2 or 3 and the second factor was the inclusion level of an α-AN spike, 0, 0.28 mg g^{-1} soya or 0.56 mg g^{-1} soya.

Raw soyabeans were ground in a hammer mill through a 1.5mm screen. The ground beans were mixed well to distribute particle sizes evenly and weighed into 18 x 0.25 g aliquots into centrifuge tubes. To each of 6 tubes 5 ml distilled water was added, a second set of 6 tubes each had 4.5 ml distilled water added and 0.5 ml 0.01M glycine, the final set of 6 tubes had 4 ml distilled water and 1 ml 0.01M glycine added. The 0.01M glycine spike provided 0.07 mg and 0.14 mg α-AN per 0.25 g aliquot of soyabean respectively. All

tubes were mixed for 1 min on a vortex mixer, then left to stand for 2 h. Tubes were then mixed again and centrifuged at 10 000 r.p.m. for 10 min. The supernatant was removed and retained and a further 5 ml distilled water was added to each tube. The tubes were mixed for 1 min on a vortex mixer, left to stand for 10 min, remixed and centrifuged. This procedure was repeated a third time, each time the supernatants were retained. For each sample 0.25 ml was removed from the first supernatant (S1), the first two supernatants were then pooled, mixed and 0.25 ml removed (S2). The third supernatant was added to the first two, mixed and 0.25ml removed (S3). The samples of S1, S2, and S3 were diluted 1:20, 1:10 and 1:6.67 respectively to provide a final dilution factor of 1:400 with respect to the initial aliquot of ground soyabean. Each sample was assayed in triplicate for α-AN according to the A.O.A.C. (1992) method, using the calculation from equation 1. The data were checked for normal distribution and analysed by analysis of variance using Minitab (Minitab inc. release 10.2, State College, Pennsylvania, 1994). Treatment means were compared using Newman-Keuls multiple range test.

2.3.3. *Results.*

The results of the α-AN assay are presented in Table 2.3.1. The data in this table show that there was a significant ($P<0.001$) difference in α-AN content between the spiked and unspiked samples and between the two levels of spiked samples for all extraction procedures. The first extraction, S1, produced significantly ($P<0.001$) lower α-AN levels than either of the pooled extraction increments, S2 and S3. There was no significant difference between α-AN levels in S2 and S3 regardless of the level at which the samples had been spiked. The α-AN spike was not fully recovered in any of the samples. The recovery rate of the 0.28 mg spike was 97 %. 94 % and 83 % for extractions 1, 2 and 3 respectively and the recovery rate of the 0.56 mg spike was 90 %, 90 % and 89 % for extractions 1, 2 and 3 respectively.

Table 2.3.1 Factorial analysis of mean α-amino nitrogen (mg g^{-1} soya) in spiked and unspiked samples of ground raw soyabean for three incremental extraction steps.

extraction	spike (mg α-AN g^{-1} soya) 0	0.28	0.56	main effect extraction	s.e.d.
S1	0.57	0.87	1.07	0.82	0.006
S2	0.73[a]	0.99[a]	1.23[a]	0.98[a]	
S3	0.74[a]	0.97[a]	1.23[a]	0.98[a]	
main effect spike	0.68	0.93	1.18		
s.e.d.	0.006				

[a] means within the same column with the same superscript are not significantly different ($P > 0.05$)
s.e.d. of the interaction = 0.010

2.3.4. *Discussion.*

The results seem to suggest that all of the recoverable α-AN from the spike was recovered in the first extract. The percentage recovery of α-AN in the spiked samples decreased with subsequent extraction steps indicating that the extra α-AN extracted by these steps originated from the ground soya rather than the spike.

In this study more care was taken in timing of the heating step of the method. Tubes were heated in batches of 6 rather than 15 and this enabled more accurate timing. The coefficient of variation ranged from 1.2 % to 4.3 % for sample replicates and was 1.2 % for the standards. In this experiment the ground soyabean was not mixed with water prior to taking the samples for extraction, thus allowing a more homogenous sample to be taken compared with the previous experiment. However, in subsequent experiments, in which the effect of protease pretreatment of soyabean slurries was to be investigated, samples would have to be taken from slurries. Therefore, the effect of particle size on the extraction of α-AN and a suitably reliable sampling technique needed to be established.

From this study it was concluded that two extraction steps were necessary to extract the maximum amount of soluble protein and hence α-AN. As there was no significant difference between the amount of α-AN in the second and third extractions it was felt that there was little to be gained by using three extraction steps taking into account the amount of time this would require when processing large numbers of samples. Therefore, in all subsequent studies where extraction of α-AN was performed this was done by pooling the supernatants from the first two washes.

2.4 The effect of particle size and enzyme treatment on the determination of α- amino nitrogen in soyabean slurry.

2.4.1 *Introduction*

In the previous two studies it was observed that grinding whole raw soyabean in a hammer mill using a 1.5mm screen, which was the smallest screen size available, produced a product with a variety of particle sizes. When soyabean ground in this way was mixed with water to form a slurry it was very difficult to obtain subsamples which were representative of the whole product because the larger particle sizes settled out very quickly. The protein in soyabean is contained in intracellular protein bodies. Therefore, in order for the protein to be released into solution the cells of the soyabean cotyledon must be disrupted. During grinding some cell wall disruption occurs within the particle, due to the shear forces generated by the mechanical process (Sineiro, Dominguez, Nunez and Lema 1998), as well as the cells on the particle surface being fractured. The degree of cell damage will be greater in the smaller particles and the large surface area : volume ratio they present will allow a higher degree of leaching of protein into solution. It will also increase the amount of protein exposed to any exogenous enzyme added to the soyabean slurry. Therefore, it may be expected that particle size will affect the amount of soluble protein and hence the amount of α-AN extracted from ground soyabean.

The previous two studies have shown that α-AN can be detected in soyabean slurries. Any hydrolysis of soyabean protein by exogenous proteases will increase the number of terminal α- amino groups exposed at the ends of cleaved peptide bonds. This increase should be detected as an increase in α-AN.

In order to compare the proteolytic action of two proteases the inclusion levels were calculated on the basis of units g^{-1} nitrogen in soyabean. Soyabean contains approximately 5.84 % N and therefore, 1 g N equates to 17.12 g soyabean.

It is also possible that constituents of the soyabean seed such as protein and fat may be partitioned between different particle sizes.

The objectives of this study were to determine:

- the distribution of particle sizes in raw soyabean ground in a hammer mill (1.5mm screen).
- if constituents of the seed are evenly distributed between various particle sizes.
- the effect of particle size on the determination of α-AN extracted from soyabean slurry.
- the effect of particle size on the action of two exogenous proteases as determined by α-AN extracted from protease treated soyabean slurry.

2.4.2 *Materials and methods*

Two studies were conducted to examine the effects of grinding and protease treatment on the production of αAN from raw soya. The studies were designed according to a three factor factorial design. Factor 1 was the presence or absence of a protease, (0 or 1 000 units P2 g^{-1} N in study A and 0 or 1 000 units P3 g^{-1} N in study B). Factor 2 was the duration of steeping (0, 1, 2 or 3 days). Factor 3 was particle size (1 mm, 0.5 mm, 0.25 mm or 0.125 mm).

Two studies were conducted because the two proteases used in these studies had different pH optima. The proteases, P2 and P3 were supplied by FinnFeeds International Ltd. (Marlborough UK). P2 was supplied as a fine powder and had a pH optimum of pH 4 and

an activity of 466 000 units g^{-1}. P3 was supplied as a liquid and had a pH optimum of pH 6.5 and an activity of 50 000 units ml^{-1} (H. Schulze FinnFeeds International Ltd. Personal communication). Prior to conducting the studies the activity of the enzymes was checked using the same method as the suppliers (Appendix 1) to determine that there was no loss of activity during transit. The activities were 450 000 units g^{-1} and 60 000 units ml^{-1} for P2 and P3 respectively. The two studies were conducted in a manner that allowed the two control sets of samples, i.e. those with no enzyme addition, to be compared in order to ascertain the effect of steeping pH on the determination of α-AN. This was done by analysing the two 'control' data sets according to a three factor factorial design in which the factor one was steeping pH, (pH 4 or pH 6.5) factor two was particle size, (1 mm, 0.5 mm, 0.25 mm or 0.125 mm) and factor three was duration of steeping (0, 1, 2 or 3 days).

Five hundred grams of whole raw soyabeans were ground in a hammer mill through a 1.5mm screen. The resultant ground meal was collected and passed through a set of Endicott graded sieves with aperture sizes of 1 mm, 0.5 mm 0.25 mm and 0.125 mm. The particles retained in the sieves and those in the collecting tray beneath the smallest aperture sieve were collected and weighed. Although the fractions retained in each sieve contained a range of particle sizes i.e. the fraction collected in the 0.5 mm sieve contained particles ranging between 1 mm and 0.5 mm in size, they were classified according to the aperture size of the retaining sieve for clarity.

Samples of the ground soyabean particles collected in each sieve (1 mm, 0.5 mm, 0.25 mm and 0.125 mm) and a sample of the unsieved soyabean were retained for proximate analysis which was conducted according to the methods described by James (1995).

Study A

For each particle fraction (1 mm, 0.5 mm, 0.25 mm and 0.125 mm) two sets of triplicate samples of 20 g of ground soyabean were mixed with 60 ml tap water to form a slurry and adjusted to pH 4 with 4M HCl. To one set of samples 1 000 units P2 g^{-1} N were added. The slurries were incubated at 20° C and subsamples of 1g of slurry were taken daily for four days into centrifuge tubes using a spatula. Soluble protein was extracted using two extraction steps according to the method in section 2.2.2. and α-AN determined according to method used in section 2.2.2.

Study B

Study B was conducted in the same manner as study A using 1 000 units P3 g^{-1} N in the protease treated slurries. The pH of the soyabean slurries mixed with tap water was pH 6.5 which was within the optimum range for P3, therefore no pH adjustment was necessary.

All data sets were checked for normal distribution prior to analysis by analysis of covariance with the initial α-AN content as the covariate factor using Minitab (Minitab inc. release 10.2, State College, Pennsylvania, 1994). Treatment means were compared using Newman-Keuls multiple range test.

2.4.3. *Results*

Proximate analysis

The weights of the different particle size fractions in 500 g ground raw soyabean were 80.2 g, 225.9 g, 175.1 g, 18.4 g and 0.38 g for particle sizes of 1 mm, 0.5 mm, 0.25mm, 0.125 mm and <0.125 mm respectively.

The results of the proximate analysis of each particle fraction and of the unsieved ground soyabean are presented in Table 2.4.1. Crude fat and crude protein increased with decreasing particle size and crude fibre decreased with decreasing particle size in a linear manner. There was a negative correlation between crude fibre and crude fat of -0.97 and between crude fibre and crude protein of -0.75.

Table 2.4.1 Proximate analysis of sieved fractions of ground raw soyabean.

	complete	Particle size (mm)						
g kg^{-1}	sample	1	0.5	0.25	0.125	s.e.d.	linear trend y =	r^2
DM	904[a]	911[ab]	908[ab]	919[b]	917[ab]	6.3	$+8.3x10^{-4}$size+8.2	0.13
as g kg^{-1} DM								
Crude protein	415[a]	400[b]	406[ab]	415[a]	420[a]	6.5	$-2.2x10^{-3}$size+42.0	0.53
Crude fat	127	64	111	165[a]	198	5.8	$-1.5x10^{-2}$size+20.4	0.93
Crude fibre	82	173	126	45	14	7.0	$+1.8x10^{-2}$size+0.5	0.91
Ash	21[a]	25[a]	27[a]	31[a]	29[a]	6.2	$-7.3x10^{-4}$size+3.2	0.12
N.F.E	356[a]	339[a]	330[a]	344[a]	339[a]	11.6	$-1.1x10^{-3}$size+25.6	0.05

[ab] means within the same row with the same superscript are not significantly different

α-amino nitrogen assay.

The results of the factorial analysis of the effect of pH, particle size and duration of steeping on the production of α-AN in the control samples are presented in Tables 2.4.2.a.-c. The data in Table 2.4.2 show that steeping pH, particle size and duration of steeping all significantly affected α-AN production. Steeping at pH 4 significantly reduced ($P < 0.001$) α-AN production by 0.37 (s.e.d. 0.01) mg g^{-1} soya compared to steeping at pH 6.5. This effect was apparent in all particle sizes. α-AN production significantly increased ($P < 0.001$) as particle size was reduced. There were incremental increases in α-AN

production of 0.36, 0.14 and 0.16 (s.e.d. 0.014) mg g^{-1} soya as particle size was reduced from 1 mm to 0.5mm, from 0.5 mm to 0.25 mm and from 0.25 mm to 0.125 mm respectively. There was a significant increase ($P < 0.001$) in α-AN production over the three day steeping period of 0.75 (s.e.d. 0.014) mg g^{-1} soya. The increase in α-AN production due to steeping time was greatest in the slurries steeped at pH 6.5 which gave an increase of 1.9 mg α-AN g^{-1} soya compared to 0.47 (s.e.m. 0.014) mg α-AN g^{-1} soya in the samples steeped at pH 4 over the three days. There was an overall significant interaction ($P < 0.001$) effect between duration of steeping and particle size. However, when the means were compared there was no significant difference between the 0.25 mm and 0.125 mm after 3 days steeping. The interactions between particle size, steeping pH and duration of steeping are presented in Table 2.4.3. Although there was a significant ($P < 0.001$) interaction effect between the three factors, there was no significant difference in α-AN production between particle sizes of 0.25 mm and 0.125 mm after 2 days of steeping or between particle sizes of 0.5 mm, 0.25 mm or 0.125 mm after 3 days of steeping at pH 6.5. There was no significant difference in α-AN production between particle sizes of 0.25 mm and 0.125 mm after 3 days of steeping at pH 4.

The factorial analysis of α-AN production in response to P2 addition is presented in Tables 2.4.4.a.-c. There were significant differences in α-AN production due to duration of steeping, particle size and P2 treatment. Treatment of the soya slurries with P2 resulted in a significant increase ($P < 0.001$) of 0.27 (s.e.d. 0.008) mg α-AN g^{-1} soya. Over the three days α-AN production was increased by P2 treatment by 0.09, 0.12, 0.29 and 0.57 (s.e.d. 0.017) mg g^{-1} soya on days 0, 1, 2 and 3 respectively. As particle size decreased there was a significant increase ($P < 0.001$) in α-AN production from 1.04 mg g^{-1} soya in the 1 mm

particle size fraction to 1.32, 1.46 and 1.73 (s.e.m. 0.008) mg g^{-1} soya in the 0.5 mm, 0.25 mm and 0.125 mm particle sizes respectively.

Analysis of the interaction between all three factors are presented in Table 2.4.5. This data showed that pretreatment of soya with P2 significantly ($P < 0.05$) increased α-AN production over the total period by 0.48, 0.73, 0.52 and 0.57 (s.e.d.0.033) mg g^{-1} soya in the 1 mm, 0.5 mm, 0.25mm and 0.125 mm particle sizes respectively compared to the untreated samples.

The factorial analyses of the data for the effect of steeping raw soya in the presence or absence of P3 is presented in Table 2.4.6.a.-c. As with study A, duration of steeping, particle size and protease treatment all significantly affected α-AN production. Treatment of the slurries with P3 significantly increased α-AN production by 0.79 (s.e.d. 0.008) mg g^{-1} soya. This effect was apparent in all particle sizes with α-AN production increasing by 0.56, 0.63, 1.0 and 0.99 (s.e.m. 0.008) mg g^{-1} soya in particle sizes 1 mm, 0.5 mm, 0.25 mm and 0.125 mm respectively due to treatment with P3. α-AN production increased with decreasing particle size, from 1.49 mg g^{-1} soya in the 1 mm particle fraction to 2.02, 2.29, and 2.41 (s.e.d. 0.016) mg g^{-1} soya in the 0.5 mm, 0.25 mm and 0.125 mm particle fractions. There was a significant ($P < 0.001$) interaction effect between particle size and duration of steeping. However, there was no significant difference in α-AN production between particle sizes of 0.25 mm and 0.125 mm after 2 or 3 days of steeping. Analysis of the interaction between all three factors showed that over the three day steeping period treatment with P3 increased α-AN production by 0.85, 0.94, 1.28, and 1.48 (s.e.m. 0.032) mg g^{-1} soya in the 1 mm, 0.5 mm, 0.25 mm and 0.125 mm particle size fractions respectively compared to the untreated controls.

Table 2.4.2. Factorial analysis of adjusted means for α amino nitrogen (mg g^{-1} soya) production in ground raw soya of different particle sizes steeped at pH 4 or pH 6.5 for 3 days at 20°C.

a) interaction between steeping time and particle size

	Particle size (mm)					
Steeping Time (days)	1.0	0.5	0.25	0.125	Main effect: Time	s.e.d.
0	0.70	0.94	1.12	1.39	1.04	0.014
1	0.93	1.21	1.31	1.62	1.27	
2	1.23	1.68	1.85	1.95[l]	1.68	
3	1.37	1.86	1.96[a]	1.97[al]	1.79	
Main effect: size	1.06	1.42	1.56	1.73		
s.e.d.	0.014					

s.e.d. for the interaction between particle size and steep time = 0.028

b) interaction between steeping time and pH

	Steeping Time (days)					
pH	0	1	2	3	Main effect: pH	s.e.d.
4	1.01	1.18	1.36	1.48	1.26	0.010
6.5	1.07	1.36	1.99	2.97	1.63	
Main effect: Time	1.07	1.27	1.68	1.79		
s.e.d.	0.014					

s.e.d. for the interaction between steep time and pH = 0.020

c) interaction between particle size and pH

	Particle size: mm					
pH	1.0	0.5	0.25	0.125	Main effect: pH	s.e.d.
4	0.99	1.17	1.34	1.52	1.26	0.010
6.5	1.12	1.67	1.78	1.94	1.63	
Main effect: size	1.06	1.42	1.56	1.73		
s.e.d.	0.014					

s.e.d. for the interaction between particle size and pH = 0.020

[a] means in the same row with the same superscript are not significantly different ($P > 0.05$)

[l] means in the same column with the same superscript are not significantly different ($P > 0.05$)

Table 2.4.3. Adjusted means for α amino nitrogen production (mg g^{-1} soya) in different particle fractions of ground raw soyabean steeped over 3 days at 20° C at pH 4 or pH 6.5.

	pH 4					pH 6.5				
	Particle size (mm)					Particle size (mm)				
Steeping time (days)	1.0	0.5	0.25	0.125	s.e.d.	1.0	0.5	0.25	0.125	s.e.d.
0	0.70^{a}	0.87	1.05^{b}	1.41^{c}	0.028	0.71^{a}	1.00^{b}	1.19	1.37^{c}	0.028
1	0.93^{a}	1.12^{b}	1.22	1.45^{c}		0.96^{a}	1.13^{b}	1.40^{c}	1.80	
2	1.14	1.26^{a}	1.45	1.59		1.31^{a}	2.09	2.23^{b}	2.31^{b}	
3	1.21	1.43	1.63^{a}	1.64^{a}		1.52	2.28^{b}	2.29^{b}	2.29^{b}	

^{a,b,c} means in the same row with the same superscript are not significantly different $P > 0.05$

the initial α amino nitrogen level in each particle fraction was used as the covariate factor in these analyses.

Table 2.4.4. Factorial analysis of adjusted means for α amino nitrogen (mg g^{-1} soya) production in ground raw soya slurries of different particle size fraction steeped for 3 days at 20° C in the presence of 0 or 1 000 units g^{-1} N of protease P2.

a) interaction between particle size and steeping time

	Particle size (mm)					
Steeping Time (days)	1.0	0.5	0.5	0.125	Main effect: Time	s.e.d.
0	0.68	0.87	1.13	1.51	1.05	0.012
1	0.92	1.16	1.24	1.62	1.24	
2	1.17	1.47	1.58	1.79	1.50	
3	1.40	1.77	1.89	1.99	1.76	
Main effect: size	1.04	1.32	1.46	1.73		
s.e.d.	0.012					

s.e.d. for the interaction between particle size and steep time = 0.024

b) interaction between protease treatment and steeping time.

	Steeping Time (days)					
Protease treatment (units g^{-1} N)	0	1	2	3	Main effect: Protease	s.e.d.
0	1.01	1.18	1.36	1.48	1.25	0.008
1 000	1.09	1.30	1.65	2.05	1.52	
Main effect: Time	1.05	1.24	1.50	1.76		
s.e.d.	0.012					

s.e.d. for the interaction between steeping time and protease treatment = 0.017

c) interaction between protease treatment and particle size

	Particle size (mm)					
Protease treatment (units g^{-1} N)	1.0	0.5	0.25	0.125	Main effect: Protease	s.e.d.
0	0.94	1.15	1.34	1.58	1.25	0.008
1 000	1.14	1.49	1.58	1.87	1.52	
Main effect: Size	1.04	1.32	1.46	1.72		
s.e.d.	0.012					

s.e.d. for the interaction between particle size and protease treatment = 0.017

Table 2.4.5. Adjusted means for α amino nitrogen production (mg g^{-1} soya) in different particle fractions of ground raw soyabean steeped over 3 days at 20° C in the presence or absence of 1 000 units g^{-1} N protease P2.

	No protease treatment					Treated with 1 000 units P2 g^{-1} N				
	Particle size (mm)					Particle size (mm)				
Steeping time (days)	1.0	0.5	0.25	0.125	s.e.d.	1.0	0.5	0.25	0.125	s.e.d.
0	0.64[a]	0.85[b]	1.05	1.47[c]	0.033	0.71[a]	0.89[b]	1.21	1.54[c]	0.033
1	0.87	1.09	1.23[a]	1.21[a]		0.97	1.23[a]	1.26[a]	1.74	
2	1.09	1.24[a]	1.46	1.65[b]		1.24[a]	1.72[b]	1.70[b]	1.93	
3	1.16	1.40	1.63[a]	1.70[a]		1.64[a]	2.13[b]	2.15[b]	2.27	

[a,b] means in the same row are not significantly different. $P > 0.05$

* the initial α amino nitrogen level in each particle fraction was used a the covariate factor in these analyses.

Table 2.4.6. Factorial analysis of adjusted means for α amino nitrogen (mg g^{-1} soya) production in ground raw soya slurries of different particle size fraction steeped for 3 days at 20° C in the presence of 0 or 1 000 units g^{-1} N of protease P3.

a) interaction between particle size and steeping time

	Particle size (mm)					
Steeping Time (days)	1.0	0.5	0.25	0.125	Main effect: Time	s.e.d.
0	0.81	1.02	1.24	1.41	1.12	0.016
1	1.32	1.85	2.20	2.42	1.95	
2	1.78	2.46	2.78[a]	2.83[a]	2.46	
3	2.03	2.75	2.94[a]	3.01[a]	2.68	
Main effect: size	1.49	2.02	2.90	2.41		
s.e.d.	0.016					

s.e.d. for the interaction between steep time and particle size = 0.024

b) interaction between protease treatment and steeping time.

	Steeping Time (days)					
Protease treatment (units g^{-1} N)	0	1	2	3	Main effect: Protease	s.e.d.
0	1.09[1]	1.40	2.01	2.12	1.66	0.011
1 000	1.14[1]	2.50	2.91	3.24	2.45	
Main effect: Time	1.12	1.95	2.46	2.68		
s.e.d.	0.016					

s.e.d. for the interaction between steeping time and protease treatment = 0.023

c) interaction between protease treatment and particle size

	Particle size (mm)					
Protease treatment (units g^{-1} N)	1.0	0.5	0.25	0.125	Main effect: Protease	s.e.d.
0	1.21	1.71	1.79	1.92	1.66	0.008
1 000	1.77	2.34	2.79	2.91	2.45	
Main effect: Size	1.49	2.02	2.29	2.41		
s.e.d.	0.011					

s.e.d. for the interaction between particle size and protease treatment = 0.023

[a] means in the same row with the same superscript are not significantly different ($P > 0.05$)

[1] means in the same column with the same superscript are not significantly different ($P > 0.05$)

Table 2.4.7. Adjusted means for α amino nitrogen production (mg g^{-1} soya) in different particle fractions of ground raw soyabean steeped over 3 days at 20° C in the presence or absence of 1 000 units g^{-1} N protease P3.

	No protease treatment					Treated with 1 000 units P3 g^{-1} N				
	Particle size (mm)					Particle size (mm)				
Duration of steeping (days)	1.0	0.5	0.25	0.125	s.e.d.	1.0	0.5	0.25	0.125	s.e.d.
0	.79[a]	1.04[b]	1.20	1.35	0.045	0.83[a]	1.00[b]	1.27	1.47	0.032
1	1.04	1.35[a]	1.40[a]	1.77		1.60	2.36	3.00[b]	3.06[b]	
2	1.39	2.13[a]	2.25[b]	2.25[b]		2.17[a]	2.78	3.31[c]	3.38[c]	
3	1.61	2.23[a]	2.30[a]	2.27[a]		2.46	3.17	3.58	3.75	

[a,b,c] means in the same row with the same superscript are not significantly different. $P > 0.05$

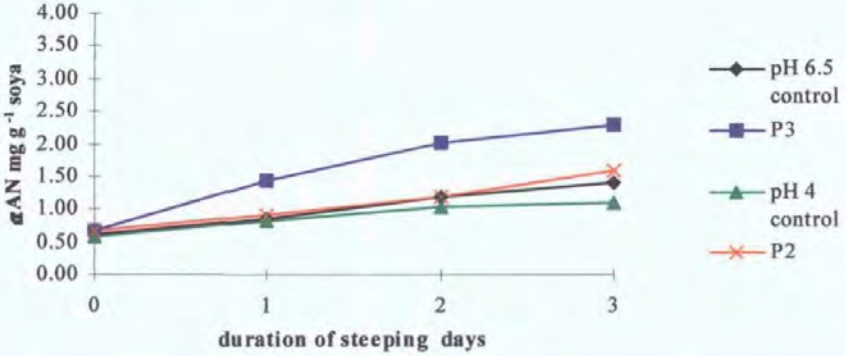
The mean α-AN production in response to protease treatment and pH of the different particle size fractions over the three day period are presented as reaction-time plots in Figure 2.4.1.a. - 2.4.1.d. These Figures show that α-AN production was still increasing after three days steeping in all particle sizes and for all treatments. The greatest increases in α-AN production were observed with P3 treatment of the 0.25 and 0.125 mm particle sizes over the first 24 h of steeping. The initial α-AN levels detected in the different particle fractions steeped at pH 6.5 varied between 0.60, 0.99, 1.24, and 1.48 (s.d. 0.065) mg g^{-1} soya for particle sizes of 1 mm, 0.5 mm, 0.25 mm and 0.125 mm respectively and from 0.58, 0.82, 1.06 and 1.53 (s.d. 0.062) mg g^{-1} soya respectively for samples steeped at pH 4.

2.4.4. *Discussion*

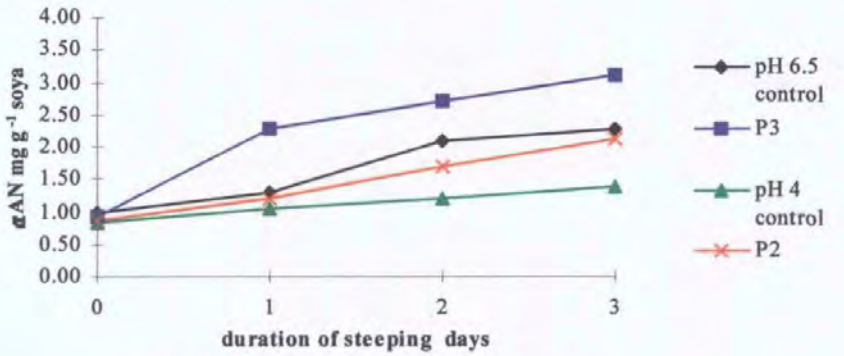
Proximate analysis

Crude fibre and crude fat were unevenly distributed between the different particle sizes. The opposing linear trends of crude fat and crude fibre, and the strong negative correlation (-0.97) between them, reflect the partitioning of crude fibre in the particle fractions. Flakes of seed coat were clearly visible in the 1 mm and 0.5 mm fractions. The value for the crude fat content of the 0.125 mm fraction was close to the accepted value of 20% crude fat for soyabean, whereas the values for the larger particle sizes were lower than expected. The crude fat content of the unsieved sample was also lower than expected and this may have reflected the range of particle sizes in this sample. Although there was a linear trend towards increasing crude protein content with decreasing particle size there was no significant difference ($P > 0.05$) between the crude protein content of the 0.5, 0.25 and 0.125 particle sizes. The significantly lower ($P<0.05$) protein content of the 1 mm fraction

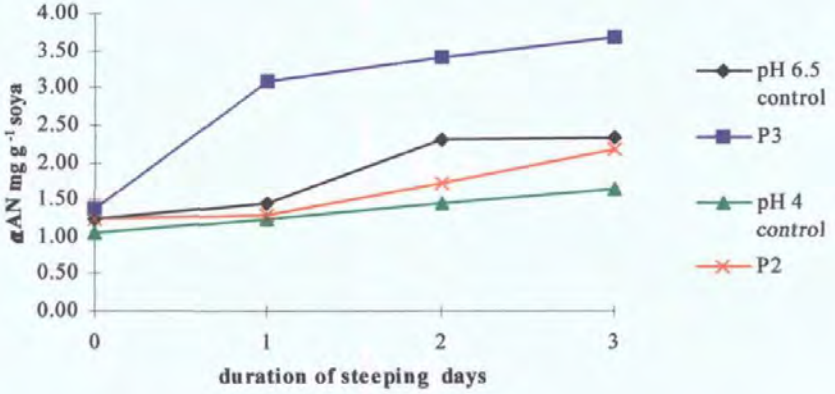
Figure 2.4.1 α-amino nitrogen production in different particle sizes of ground raw soyabean steeped over three days at pH 4 in the presence or absence of 1 000 units P2 g^{-1} N or pH 6.5 in the presence or absence of 1 000 units P3 g^{-1} N.



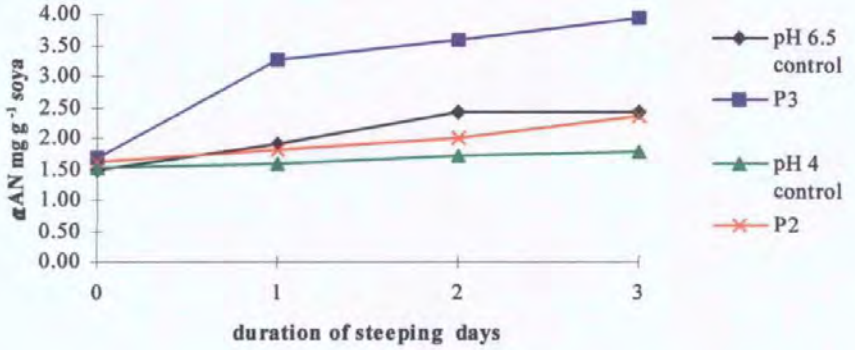
a) 1 mm particle size fraction



b) 0.5 mm particle size fraction



c) 0.25 mm particle size fraction



d) 0.125 mm particle size fraction.

was due to the proportionally larger amount of fibre in this fraction. The nitrogen free extractive component of the analyses reflects discrepancies in the measurement of crude fat and the distribution of fibre between the particle fractions.

α- amino nitrogen assay.

Analysis of the data for α-AN production in the control samples steeped at pH 4 and pH 6.5 demonstrated that pH had a significant effect on the α-AN assay. This was probably due to the effect of pH on soya protein solubility. Soya protein has minimum solubility between pH 3.75 and pH 5.25 (Pearson 1983) and it was considered likely that this affected the outcome of the α-AN assay by reducing the amount of protein in solution and hence the amount of α-AN that could be extracted. Although the extraction process used a ratio of 1g slurry:10 ml water this may not have been sufficient to overcome the effect of pH on the solubility of soyabean protein. The validity of the results obtained for soya treated with P2 must be questioned in view of the detrimental effect of steeping at pH 4, as these samples were also steeped at pH 4. There was a significant increase of 22 % (0.27 s.e.m.0.008) α-AN detected in the soya slurries treated with P2 compared with the untreated slurries, which indicated that P2 was capable of hydrolysing soya protein to a certain extent. However, this may not have been a true indication of the extent to which P2 was able to hydrolyse soya protein due to the confounding effect of pH on the extractability of α-AN.

As expected, particle size significantly affected the production of α-AN in the samples. The initial variation in α-AN extracted from the samples prior to steeping or enzyme treatment supported the view, made in the introduction to this study, that the larger surface area: volume ratio and greater degree of cell damage in the smaller particle sizes allowed a greater quantity of protein to leach into solution. The effect of particle size was also

evident in the response to P3 addition. α-AN production due to hydrolysis of soya protein by P3 was greatest in the 0.25 and 0.125 mm particle sizes.

Figure 2.4.1.b and 2.4.1.c.. show the increases in α-AN over time for the control samples at pH 6.5 of particle fractions 0.5 mm and 0.25 mm followed the same pattern as previously observed in study 2.2 (Fig. 2.1). The α-AN extracted from the 1 mm particle fraction was much lower over the time course of the study compared with the smaller particle fractions. This suggests that the cellular structure in particle sizes <0.5 mm was not sufficiently disrupted to allow full extraction of the protein into solution. In accordance with the results from study 2.2, the increase in α-AN production over three days in the control samples steeped at pH 6.5 was indicative of the presence of an endogenous protease in the raw soyabean.

The reaction-time plots for the samples treated with P3 presented in Figure 2.4.1.b, 2.4.1.c.and 2.4.1.d. show an initial increase in α-AN production over the first 24 h after which α-AN production slowed and tended to plateau. This type of relationship is typical of a reaction-time plot for enzyme catalysed reactions and slowing of the reaction rate over time is usually due to a number of contributory factors, namely, instability of the enzyme, depletion of the substrate and inhibition of the enzyme by the end products of the reaction. However, it was not possible from these data to determine the relative contributions of these factors in slowing down the rate of hydrolysis by P3. In comparison the reaction-time plot for P2 did not show the same pattern, and it was difficult to interpret the effects of P2 from this plot. As protein hydrolysis appears to have progressed more slowly it is possible that the rate of reaction did not reach a plateau level over the time course of this study. However, as pH had a detrimental effect on the extractability of α-AN the

significance of any of the results obtained from the slurries steeped at pH 4 must be questioned.

2.4.5 *Conclusions.*

The detection of α-AN appears to be an appropriate method for determination of protein hydrolysis for P3 which has a pH optimum *circa* pH 6.5. However, it does not appear to be appropriate for P2 which has a pH optimum *circa* pH 4 because of the confounding effect of low pH on protein solubility and hence on the outcome of the α-AN assay.

It was considered that in future studies it would be necessary to use ground soyabean samples of defined particle size. Unfortunately, the yield of the smallest particle fraction (0.125 mm) was very low, 18.4 g per 500g ground raw soyabean. Therefore, it was decided that in future studies the two smallest particle fractions would be pooled. Although the differences in α-AN production between these two particle fractions were significant ($P<0.001$) overall, they were not as great as the differences in α-AN between both of them and the larger particle fractions (1 mm and 0.5 mm). Therefore, to avoid reiteration, in all further studies where raw soyabean slurries are prepared, this is the particle size fraction that passes through a 0.5 mm sieve mixed with water in a ratio of 1 soyabean : 3 water.

2.5. Assessment of the microbial population in ground raw soyabeans steeped for four days at pH 6.5 and pH 4 in the presence or absence of proteases P3 and P2.

2.5.1 *Introduction.*

Over the four day time course of the previous studies it was apparent that the soyabean slurries became contaminated with microorganisms which caused them to ferment. The microorganisms probably originated from the soyabeans themselves, although contamination from other sources cannot be ruled out. The fermentation was most apparent after the first 48h of steeping, when the slurries became encrusted and gas was released on stirring. Geary (1997) demonstrated that, when compound weaner pig feed is steeped prior to use in liquid feed systems, a microbial population develops and the feed ferments. In Geary's (1997) study the predominant group of organisms in the microbial population were lactic acid bacteria, which caused a lactic fermentation and consequent pH reduction in the liquid feed. Geary (1997) concluded that the lactic fermentations had a preservative role, preventing the growth of food spoilage organisms and potential pathogens. The fermentable substrate composition of the compound feed used in Geary's (1997) study was conducive to development of a lactic fermentation. However, in substrates containing high proportions of protein, spoilage due to protein degradation can occur (Prescott *et al.* 1993). It was possible that this was occurring in the soyabean slurries in the latter part of the previous study as they emitted an unpleasant odour. Fermentation of soyabeans is a common process in the production of a range of soya foods; however, in such practices fermentation is conducted under controlled conditions and by inoculation of cooked soyabeans, usually with fungal cultures (Lin 1991).

The objective of this study was to determine and characterize the microbial population in ground steeped soyabean over a time course of four days. The pH of the soyabean slurry is

likely to affect the microbial population as many microorganisms cannot tolerate low pH's (Prescott *et al.* 1993). The effect of exogenous enzyme activity on the microbial population was also investigated as it was possible that the hydrolysis of protein could encourage microbial growth.

2.5.2 *Materials and methods.*

Three studies were conducted to determine the effect of duration of steeping, pH and protease addition on the microbial population in steeped raw soyabean slurries. The studies were conducted according to a two factor factorial design in which the first factor for all three studies was the duration of steeping, 0 - 3 days. The second factor was pH (pH 4 or pH 6.5), P2 addition (0 or 1 000 units g^{-1} N) and P3 addition (0 or 1 000 units g^{-1} N) for studies A, B and C respectively.

Raw soyabean slurries were prepared by mixing ground raw soyabean with water in a ratio of 1:3 as in previous studies. Three replicates of each of the following treatments were prepared:-

CA mixed with water only

P3A mixed with water plus 1 000 u g^{-1} N of P3

CB mixed with water and pH adjusted to pH 4 with 4M HCl

P2B mixed with water and pH adjusted to pH 4 with 4M HCl plus 1 000 u g^{-1} N of P2

The slurries were steeped for three days and a 1 ml subsample was taken from each daily for microbial assessment which was conducted as follows. Samples were serially diluted (10 fold dilutions) in one quarter strength Ringers solution (Unipath Ltd. Basingstoke, Hampshire) to a dilution of 10^{-5} on day zero and 10^{-8} on subsequent days. Appropriate dilutions were plated and incubated to assess the microbial population as follows. Total viable aerobes and lactic acid bacteria were assessed using the pour plate method (Banwart

1989) on Plate Count Agar (PCA) (Unipath Ltd.), incubated aerobically at 30° C for 3 days and de Mann, Rogosa, Sharpe Agar (MRS) (Unipath Ltd.), incubated anaerobically at 37° C for 2 days respectively. Coliform bacteria and yeasts were assessed using the surface plate count method (Banwart 1989) on MacConkey Agar (MCA) (Unipath Ltd.), incubated aerobically at 37° C for 24h and on Rose Bengal Chloramphenicol Agar (RBCA) (Unipath Ltd.), incubated at 22° C for 5 days respectively. The pH of each slurry sample was measured daily using a pH meter (EIL 7015, Kent) fitted with a Gelplas electrode (BDH Ltd.). A selection of the predominant colony types was examined microscopically using Grams stain according to the standard method (Banwart 1989) and the catalase activity of these organisms was determined by emulsifying colonies in hydrogen peroxide on a microscope slide and examining for gas production.

The results were analysed statistically by analysis of variance using Minitab (Minitab inc. release 10.2, State College, Pennsylvania, 1994).

2.5.3 *Results*

The results of the factorial analysis of study A are presented in Table 2.5.1. Coliform bacteria were not included in this analysis as none were isolated from the samples steeped at pH 4, and very few from the samples steeped at pH 6.5. The detection limit of the surface plate count was 10 c.f.u. ml^{-1} and although one or two colonies grew on some of the MacConkey plates the mean colony count per sample was < 10 c.f.u. ml^{-1} slurry. There was a significant ($P < 0.001$) effect on microbial numbers due to pH. Steeping at pH 4 compared to pH 6.5 reduced the total viable count by 1.3×10^6 (s.e.d. 0.1 (log_{10})) c.f.u. ml^{-1} and lactic acid bacteria from 2.5×10^5 (s.e.d. 0.1 (log_{10})) c.f.u. ml^{-1} and increased the number of yeasts by 8.6×10^4(s.e.d. 0.013 (log_{10})) c.f.u. ml^{-1}.

Table 2.5.1. Factorial analysis of the main effects of the duration of steeping and steep pH on the microbial population (log_{10} c.f.u. ml^{-1}) in ground raw soyabean steeped at 20° C.

	Duration of steeping days					pH		
	0	1	2	3	s.e.d.	4	6.5	s.e.d.
Total aerobes (c.f.u ml^{-1})	4.17	4.74	6.90	7.61	0.10	5.13	6.57	0.08
Lactic acid bacteria (c.f.u. ml^{-1})	2.03	3.42	6.02	6.91	0.10	3.80	5.39	0.08
Yeasts (c.f.u. ml^{-1})	2.99	3.79	4.58	5.41	0.13	4.95	3.43	0.09

Table 2.5.2. Analysis of the interaction between pH and duration of steeping on the microbial population (log_{10} c.f.u. ml^{-1}) in raw soyabean slurries steeped at 20 ° C.

pH	4					6.5				
Steeping time (days)	0	1	2	3	s.e.d.	0	1	2	3	s.e.d.
Total aerobes (c.f.u. ml^{-1})	4.08^{a}	4.47^{a}	5.35	6.63	0.14	4.25	5.00	8.44^{b}	8.60^{b}	0.14
Lactic acid bacteria (c.f.u. ml^{-1})	2.45	3.82	4.32^{a}	4.63^{a}	0.14	1.61	3.03	7.31	9.20	0.14
Yeasts (c.f.u. ml^{-1})	3.25	4.40	5.48	6.67	0.18	2.73^{a}	3.18^{a}	3.67	4.16	0.18

[ab] means with the same superscript in the same row are not significantly different ($P > 0.05$)

The microbial numbers significantly increased over the duration of the study. Total aerobes increased by 2.7 x 10^3 (s.e.m. 0.05 (log_{10})) c.f.u. ml^{-1}, lactic acid bacteria increased by 7.7 x 10^4 (s.e.m. 0.05 (log_{10})) c.f.u. ml^{-1} and yeasts increased by 2.6 x 10^2 (s.e.m. 0.09(log_{10})) over the duration of the study.

In studies B and C the addition of P2 and P3 respectively, had no significant effect on microbial numbers (P >0.05).

The changes in the microbial populations in the slurries steeped at pH 6.5 and pH 4 over the time course of the study are presented in Table 2.5.2. and Figures 2.5.1 and 2.5.2 respectively.

The mean pH of the samples CA and P3A decreased over time from pH 6.5 on day zero to pH 6.4, pH 5.7 and pH 4.3 on days 1 to 3 respectively. The pH reduction was probably due to the production of lactic acid by LAB.

PCA is a non-selective medium that supports the growth of a wide range of microorganisms and although plate counts performed using these type of media are often termed 'total viable' counts, they do in fact only enumerate the bacteria that will actually grow on the medium in question. A diverse population of microorganisms grew on the PCA plates, with no single colonial form predominating. However, the population did appear to change over time. In the samples examined on day zero and day one a number of small regular colony forms predominated. Gram staining revealed that these were small irregular Gram positive rods, Gram positive coccobacilli and larger regular Gram positive rods. The two former types were catalase positive and the latter catalase negative. No further identification was undertaken. However, it was speculated that the catalase positive

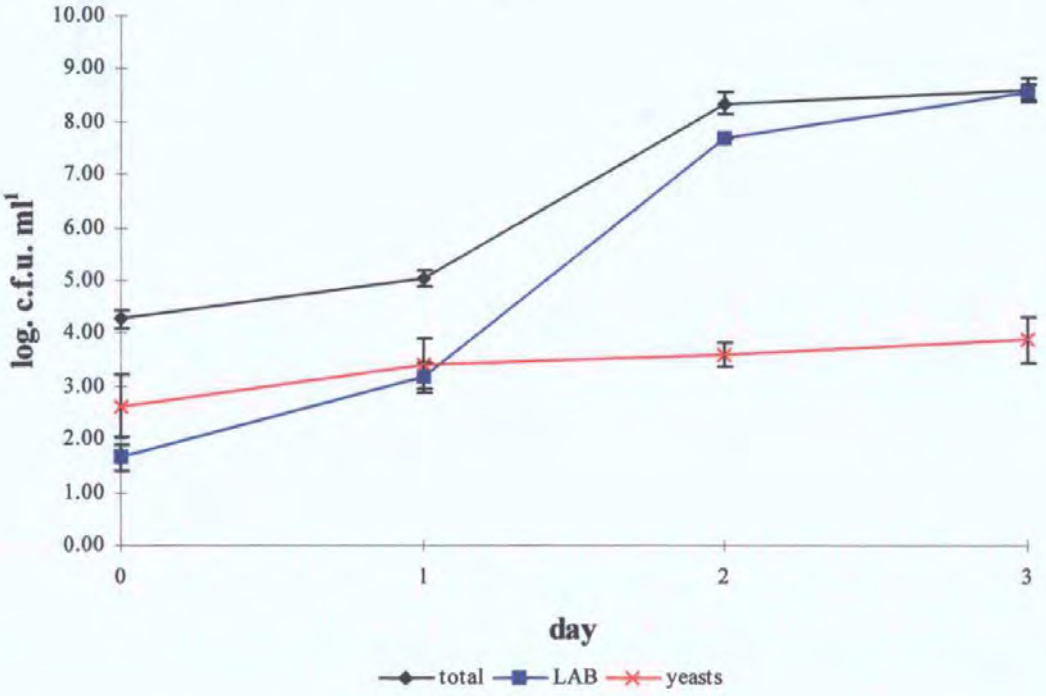


Figure 2.5.1. Log. numbers of colony forming units (c.f.u.) in soyabean slurry steeped at an initial pH of pH 6.5 showing total aerobes (total), lactic acid bacteria (LAB) and yeasts.

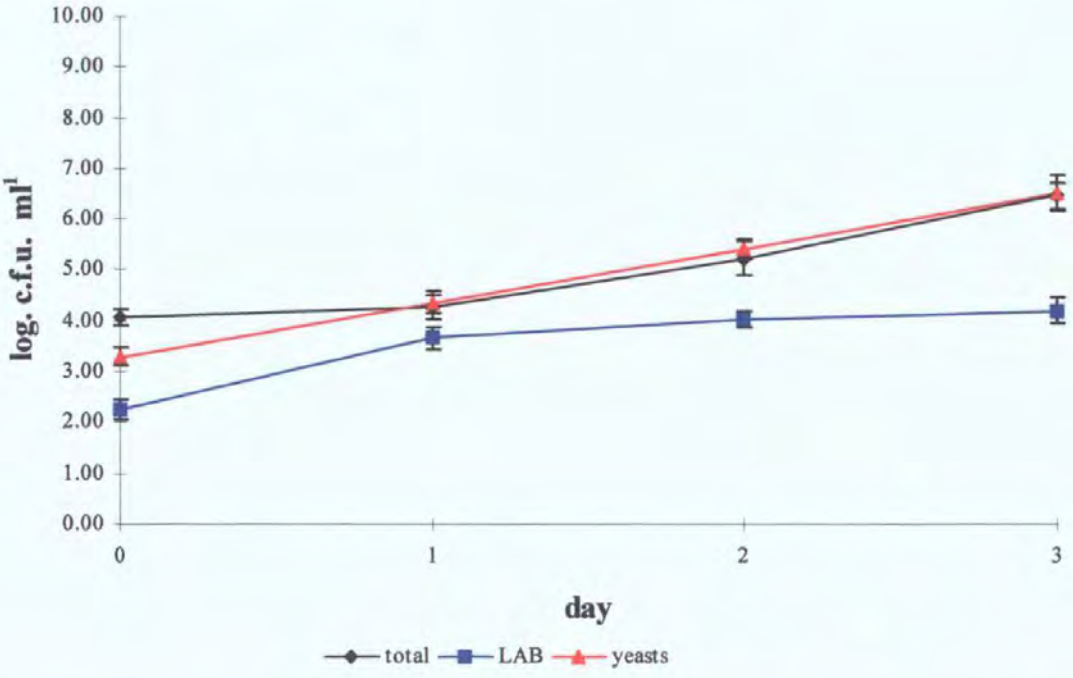


Figure 2.5.2.. Log. numbers of colony forming units (c.f.u.) in soyabean slurry steeped at pH 4, showing total aerobes (total), lactic acid bacteria (LAB), and yeasts.

bacteria could be of the genera *Corynebacterium* or *Arthrobacterium*, which are both common soil organisms, and the catalase negative bacteria could be *Lactobacilli.* In the samples examined on days two and three, large irregular colonies and filamentous colony forms predominated. Gram staining of these organisms revealed large Gram positive rods which were catalase positive and variably staining filamentous bacteria respectively. Again no further identification was undertaken. However, the colony morphology and staining characteristics of these organisms suggest that they belong to the genera *Bacillus* and *Streptomyces* respectively. The pattern of colonization was similar in the slurries steeped at pH 4; however, the numbers of colony forming units were much lower.

The other three types of media used in this study, MRS, MCA and RBCA are selective for lactic acid bacteria, coliform bacteria and yeasts and moulds respectively. Therefore no further identification was undertaken on the microorganisms which grew on these media. The coliform counts were variable in samples CA and P3A and this reflects the detection limits of the method used. For the surface plate count method a sample size of 0.1 ml is spread over the surface of an agar plate. Therefore if coliforms are present in very low numbers, < 10 c.f.u ml^{-1}, they may go undetected. Likewise coliforms may have been present in very low numbers in the slurries steeped at pH 4.

2.5.4. *Discussion.*

The increase in the number of yeasts in the slurries steeped at pH 4 reflects the ability of these organisms to tolerate low pH and take advantage of the lack of competition from otherwise faster growing bacteria. In the slurries steeped at pH 4 yeast showed a linear growth over the four day period and became the dominant type of organism. The yeast count exceeded 100 000 c.f.u. ml^{-1} by day two. Such levels of yeast contamination (> 10^5 c.f.u g^{-1}) in liquid feed for pigs can cause problems such as loss of dry matter, high gas

production and feed intake problems (G. van den Broek, Selko B.V, Tilburg, NL, personal communication).

The rapid increase in numbers of aerobes and lactic acid bacteria between days 1 and 2 in the slurries steeped at pH 6.5 represent the exponential phase of growth of these bacterial species. As the data in Figure 2.5.1 are plotted on a logarithmic scale this represents a 1000 fold and 100 000 fold increase in numbers of aerobes and lactic acid bacteria respectively. Although controlled lactic fermentations are commonly used as a means of preserving food, in this case the fermentation was essentially uncontrolled and depended on the wild type species of microorganisms contaminating the raw soyabean. It was considered that, as there was no means of controlling the fermentation, steeping times should be limited to 24h. The reason being that, during the first 24h of steeping the microbial population contaminating the raw soyabean appeared to remain in the lag phase of growth, and the increase in microbial numbers was relatively small. Another reason for limiting steeping time to 24h was the reduction in pH which occurred in the slurries after one day. This meant that after the first day P3 may have been acting under suboptimal conditions of pH. Another factor that could impinge on this was the fact that soyabean protein becomes increasingly insoluble between pH 5.25 and pH 3.75. Not only may this affect the action of the exogenous protease by limiting the availability of scissile bonds but also the exractability of α-AN is lower at low pH. Indeed this provides another explanation for the plateau effect seen in the reaction-time plot for P3 in the previous study. As pH falls, not only does the protein become insoluble, but it may also be outside of the optimum pH range of the enzyme.

2.6 Determination of enzyme inclusion levels to achieve a maximum level of protein hydrolysis in soyabean slurries in 24 h.

2.6.1 *Introduction*

The data from the previous studies indicated that a degree of protein hydrolysis was achieved in steeped soyabean slurries when exogenous proteases were added to the steep liquor. However, steeping for more than 24 to 48 h resulted in high levels of microbial contamination and fermentation of the slurries. Although feeding fermented feeds to pigs may have advantages it was felt that the development of methods to control the fermentation was outside the remit of this project. Therefore, it was decided that it would be desirable for maximum enzyme activity to occur within the first 24 h of steeping, to reduce the problem of microbial contamination.

Studies 2.3 and 2.4 showed that when α-AN levels were used as a measure of protein hydrolysis they tended to plateau after two to three days of steeping. This suggests that protein hydrolysis had approached a maximum level by this time. As the rate of an enzyme reaction is proportional to the amount of enzyme present it should be possible to increase the reaction rate by increasing enzyme concentration in order to achieve maximum hydrolysis in a shorter time.

An additional enzyme, protease P4 had been supplied by Finnfeeds International Ltd. and was included in this study. The information supplied by the manufacturers indicated that it had an optimum pH *circa* pH 6 - pH 7 and an activity of 255 416 units ml^{-1}. The activity was checked and found to be approximately 250 000 units ml^{-1}.

The objective of this study was to determine the protease inclusion level needed to achieve maximum protein hydrolysis in soyabean slurries steeped for 24 h.

2.6.2 *Materials and methods.*

A study was conducted to examine the effect of protease inclusion levels on the production of α-AN in raw soyabean slurries. The study was conducted according to a three factor factorial design, in which the first factor was duration of steeping (1, 6, 18 or 24 hours), the second factor was protease addition (P2, P3 or P4) and the third factor was protease inclusion level (0, 5 000, 10 000 or 20 000 units g^{-1} N).

In this study the method for α-AN determination used in earlier studies was adapted to allow the heating and cooling stages to take place in a thermoclycler (Progene Techne, Cambridge, UK). The advantages of using this equipment were that heating and subsequent cooling of reagent tubes could be exactly timed and twenty samples could be processed simultaneously. However, the maximum temperature that the reactants could be raised to was 99° C, whereas the A.O.A.C (1992) method states that reactants should be heated to 100° C. The use of a thermocycler also necessitated the quantities of sample and reactants to be reduced to fit into 0.5 ml Eppendorf tubes. Therefore, sample and ninhydrin reagent volumes were reduced to 0.25 ml 0.125 ml respectively. The method was validated using a series of standards against the A.O.A.C method. The full method and results of this validation study are presented in Appendix 2. The α-AN standards and the reagent blanks gave slightly lower absorbance readings when the thermocycler was used to heat the reagent tubes compared with a boiling water bath. However, the results were much less variable. As the objective of this study was to determine a threshold enzyme inclusion level and time above which α-AN levels do not increase rather than determine absolute values it was considered that its use was justifiable.

Ground raw soyabean slurries were prepared as described in section 2.4.3., and subjected to the treatments presented in Table 2.6.1. Three replicate samples of each treatment were prepared. All slurries were incubated at 20° C for 24 h.

Table 2.6.1. Identification codes and treatments of ground raw soyabean slurries used in study 2.6.

Code	pH	Protease	Inclusion level (units g^{-1} N)
C1	6.5	0	0
$P3_5$	6.5	P3	5 000
$P3_{10}$	6.5	P3	10 000
$P3_{20}$	6.5	P3	20 000
$P4_5$	6.5	P4	5 000
$P4_{10}$	6.5	P4	10 000
$P4_{20}$	6.5	P4	20 000
C2	4	0	0
$P2_5$	4	P2	5 000
$P2_{10}$	4	P2	10 000
$P2_{20}$	4	P2	20 000

Subsamples of 1 ml of each slurry were taken at 1h, 6h, 18h and 24h after addition of the enzyme. α-AN was extracted from the subsamples using the method outlined in section 2.3.4. Prior to extraction subsamples of C2, $P2_5$, $P2_{10}$ and $P2_{20}$ were adjusted to pH 6.5 with 1M NaOH. It was hoped that this would overcome the problems encountered in study 2.4 regarding the extraction α-AN from samples steeped at pH 4. α-AN content of extracted samples was determined using the method outlined above in which the heating and cooling stages were conducted in a thermocycler.

In order to determine if the problems observed with extracting α-AN from samples steeped at pH 4 had been overcome by adjusting the pH to pH 6.5 prior to extraction, a data set was

compiled from the results for the control samples C1 and C2. This data set was analysed as a two factor factorial design in which the first factor was duration of steeping and the second factor was pH.

The results were analysed statistically by analysis of variance using Minitab (Minitab inc. release 10.2, State College, Pennsylvania, 1994). All data sets were checked for normal distribution prior to analysis and treatment means were compared using Newman-Keuls multiple range test (Zar 1999).

2.6.3 *Results.*

The results for the analysis of the control samples C1 and C2 are presented in Table 2.6.2. There was no significant difference ($P > 0.05$) due to the pH of steeping. Steeping time had a significant effect on α-AN production after 6 h steep time had elapsed. There was a significant ($P < 0.001$) increase in α-AN production of 0.32 (s.e.d. 0.007) mg g^{-1} soya between the 6 h and 24 h sampling times.

Table 2.6.2 Factorial analysis of αamino nitrogen (mg g^{-1} soya) production in ground raw soya steeped at pH 6.5 or steeped at pH 4 and adjusted to pH 6.5 immediately prior to extraction.

	Steeping Time					
pH	1	6	18	24	main effect pH	s.e.d.
4	0.82	0.84[a]	0.94[a]	1.17[a]	0.94	0.007
6.5	0.83	0.85[a]	0.95[a]	1.18[a]	0.95	
main effect: Time	0.83	0.86[a]	0.95[a]	1.18[a]		
s.e.d.	0.010					

[a] means in the same row with the same superscript are significantly different ($P < 0.001$)
s.e.d. of the interaction between time and pH = 0.014

As there was no significant difference in α-AN production in the slurries steeped at pH 4 compared to those steeped at pH 6.5, it was concluded that adjusting the pH of the slurry to pH 6.5 immediately prior to extraction overcame the effect of steep pH on the extraction of α-AN. Therefore the results for treatment with P2 were included in the data set for P3 and P4 treatments.

The results of the factorial analyses for α-AN production in response to treatment with different inclusion levels of P2, P3 or P4 are presented in Tables 2.6.3.a -c. Steeping time, protease treatment and protease inclusion level all significantly increased α-AN production in the soya slurries. The main effect of steeping time was to significantly increase ($P <$ 0.001) α-AN production from 1.09 to 4.97 (s.e.m. 0.022) mg g^{-1} soya over 24 h. When the interactions between steep time and protease addition and steep time and protease inclusion level were analysed it was apparent that the overall significance of steep time on αAn production was due both to the addition of protease and the level at which they were included. α-AN production was increased by 3.31, 3.76 and 3.76 (s.e.d. 0.055) mg g^{-1} soya by the addition of P2, P3 or P4 respectively compared to 0.35 mg g^{-1} soya in the control samples over the total steep time of 24 h. Inclusion levels of 5 000, 10 000 or 20 000 units protease g^{-1} N produced increases in α-AN production of 3.99, 4.69 and 5.41 (s.e.d. 0.032) mg α-AN g^{-1} soya compared to 0.35 mg g^{-1} soya in the control samples over 24 h steep time. The best overall response to protease treatment was observed with P3, which produced 3.18 mg α-AN g^{-1} soya compared to 2.51, 2.89 and 0.95 (s.e.m. 0.019) mg g^{-1} soya for P2, P4 and the control respectively. Incremental increases in α-AN production of 1.77, 0.85 and 0.63 (s.e.d. 0.032) were observed as inclusion levels increased from 0 - 5 000, 5 000 - 10 000 and 10 000 - 20 000 units g^{-1} N respectively.

The effects of steep time and inclusion level on individual protease treatments are presented in Table 2.6.3 and Figures 2.6.1 - 2.6.3. Figure 2.6.4. shows the data from the slurries steeped for 24h as a function of enzyme concentration.

The data presented in Table 2.6.4 shows that there was no significant increase in α-AN production in the slurries treated with 20 000 units g^{-1} N of P3 or P4 after 18 h of steeping, nor was there any significant difference in α-AN production after 24 h steeping between inclusion levels of 10 000 and 20 00 units of P4. Protein hydrolysis by P2 appeared to proceed at a slower rate and even at inclusion levels of 20 000 units g^{-1} N did not completely plateau. The data plotted in Figures 2.6.2 and 2.6.3. confirm that, with inclusion levels of 20 000 units g^{-1} N, α-AN production in response to P3 or P4 reaches a plateau after 18 h steeping. The data plotted in Figure 2.6.4. show that after 24 h of steeping the there was no increase in α-AN production when P4 was included at levels above 10 000 units g^{-1} N.

2.6.4 ***Discussion.***

The data plotted in Figures 2.4.1. - 2.4.3. show that the rate of protein hydrolysis increased with increasing enzyme concentration as expected. The response curves for P3 and P4 plateau after 18h at enzyme concentrations of 20 000 units g^{-1} N indicating that maximum soya protein hydrolysis had been achieved under the conditions prevailing in the steeped slurries.

Protein hydrolysis appeared to proceed slowly over the first six hours of steeping and then increase more rapidly thereafter. This is at variance with the conventional observation that enzyme catalysed reactions proceed at a high rate initially which slows down as the substrate is depleted. However, conventional enzyme kinetics are studied with single

Table 2.6.3. Factorial analyses of α amino nitrogen (mg g^{-1} soya) production in ground raw soyabean steeped over 24 h at 20° C in the presence or absence of 0, 5 000, 10 000 or 20 000 units g^{-1} N of P2, P3 or P4

a) interaction between steeping time and protease treatment.

	Steeping Time (hours)					
Protease	1	6	18	24	Main effect: Protease	s.e.d
0	0.83[a]	0.85[a]	0.95[a]	1.18	0.95	0.027
P2	0.96[1]	1.29	3.53	4.27	2.51	
P3	1.25	1.74	4.71	5.01	3.18	
P4	1.07[1]	1.43	4.22	4.83	2.89	
Main effect: Time	1.09	1.48	4.16	4.79		
s.e.d.	0.032					

s.e.d. of the interaction = 0.055

b) Interaction between inclusion level and protease treatment

	Protease inclusion level (units g^{-1} N)					
Protease	0	5 000	10 000	20 000	Main effect: Protease	s.e.d.
P2	0.94[1]	2.33	2.93	3.86	2.51	0.027
P3	0.95[1]	3.07	3.97	4.71	3.18	
P4	0.95[1]	2.77	3.80	4.01	2.89	
Main effect: level	0.95	2.72	3.57	4.20		
s.e.d.	0.032					

s.e.d. of the interaction = 0.055

c) Interaction between steeping time and protease inclusion level.

	Steeping Time (hours)					
Inclusion level (units g^{-1} N)	1	6	18	24	Main effect: level	s.e.d.
0	0.83[1a]	0.85[a]	0.95[a]	1.18	0.95	0.032
5 000	0.97[1]	1.30	3.66	4.96	2.72	
10 000	1.18	1.78	5.45	5.87	3.57	
20 000	1.40	2.01	6.57	6.81	4.20	
Main effect: Time	1.09	1.48	4.16	4.70		
s.e.d.	0.032					

s.e.d. of the interaction = 0.063

[a] means in the same row with the same superscript are not significantly different ($P > 0.05$)

[1] means in the same column with the same superscript are not significantly different ($P > 0.05$)

Table 2.6.4. The effect of steep time and protease inclusion level on the production of α-amino nitrogen (mg g^{-1} soya) in ground raw soya slurries steeped for 24 h at 20 ° C in the presence of 0, 5 000, 10 000 or 20 000 units g^{-1} N of proteases P2, P4 or P4

	Protease inclusion level (mg g^{-1} N)												
	P2				P3				P4				
Steep Time (hours)	0	5 000	10 000	20 000	0	5 000	10 000	20 000	0	5 000	10 000	20 000	s.e.d.
1	0.81^{a1}	0.83^{a}	0.89^{a}	1.32^{cd}	0.83^{a1}	1.09^{ab}	1.50^{d}	1.56^{d}	0.83^{a1}	0.98^{ab1}	1.13^{bc}	1.31^{cd}	0.11
6	0.84^{a1}	1.23^{b}	1.38^{bc}	1.70^{d}	0.85^{a1}	1.61^{cd}	2.20^{e}	2.29^{e}	0.85^{a1}	1.06^{a1}	1.76^{d}	2.04^{e}	
18	0.94^{a12}	2.79	4.49^{b}	5.91^{c}	0.95^{a12}	4.49^{b}	5.91^{c}	7.49^{1}	0.95^{a12}	3.70	5.94^{c}	6.30^{1}	
24	1.17^{a2}	4.46	4.94^{b}	6.51^{c}	1.18^{a2}	5.09^{bd}	6.27^{c}	7.51^{1}	1.18^{a2}	5.34^{d}	6.35^{c}	6.40^{c1}	

$^{a, b, c, d, e}$ means in the same row are not significantly different ($P > 0.05$)

$^{1, 2}$ means in the same column are not significantly different ($P > 0.05$)

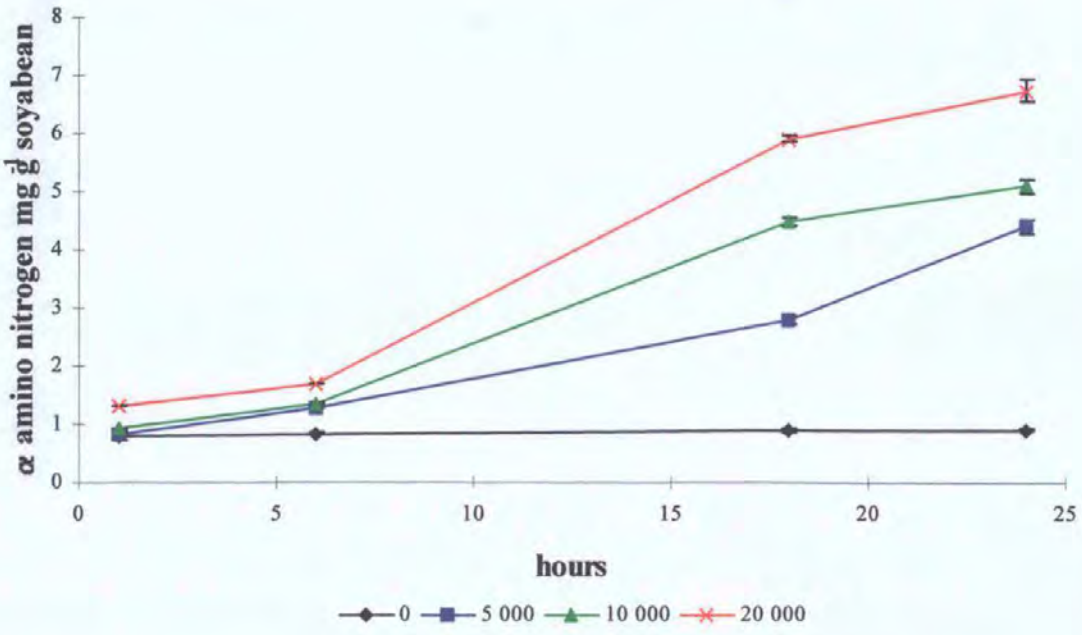


Figure 2.6.1 α-amino nitrogen levels extracted from soyabean slurry steeped for 24h at 20° C in the presence of 0, 5 000, 10 000 or 20 000 units g^{-1} N of protease P2.

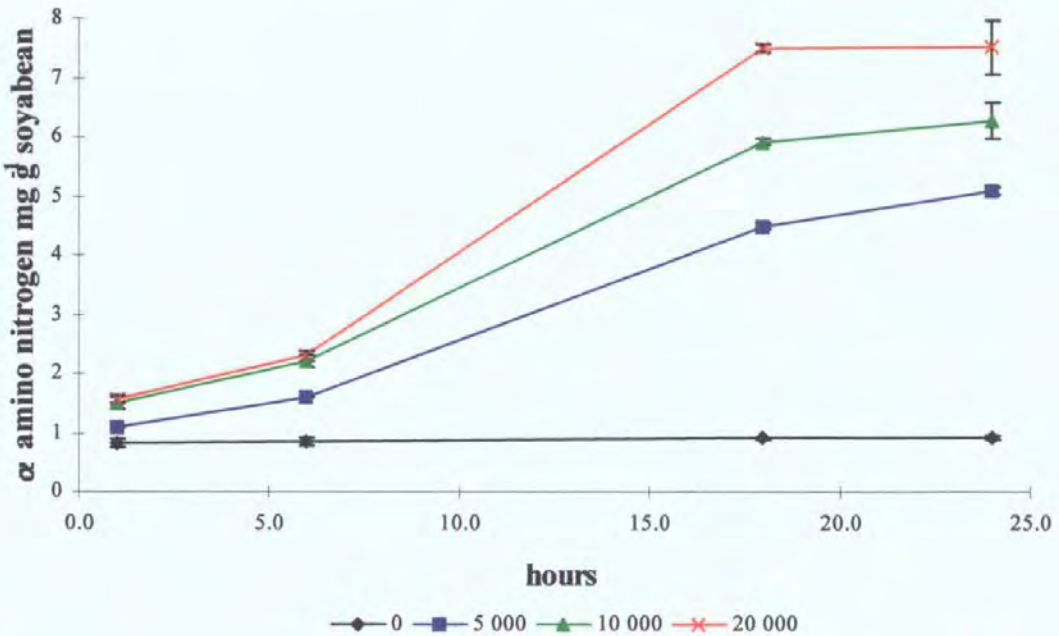


Figure 2.6.2 α-amino nitrogen levels extracted from soyabean slurry steeped for 24h at 20° C in the presence of 0, 5 000, 10 000 or 20 000 units g^{-1} N of protease P3

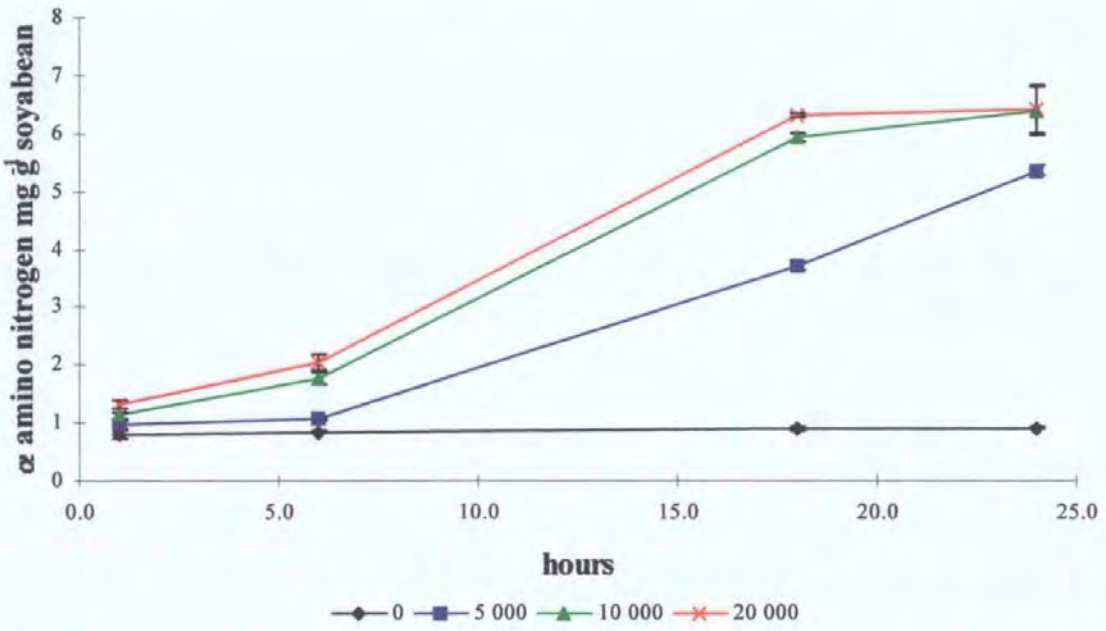


Figure 2.6.3 α-amino nitrogen levels extracted from soyabean slurry steeped for 24h at 20° C in the presence of 0, 5 000, 10 000 or 20 000 units g^{-1} N of protease P4

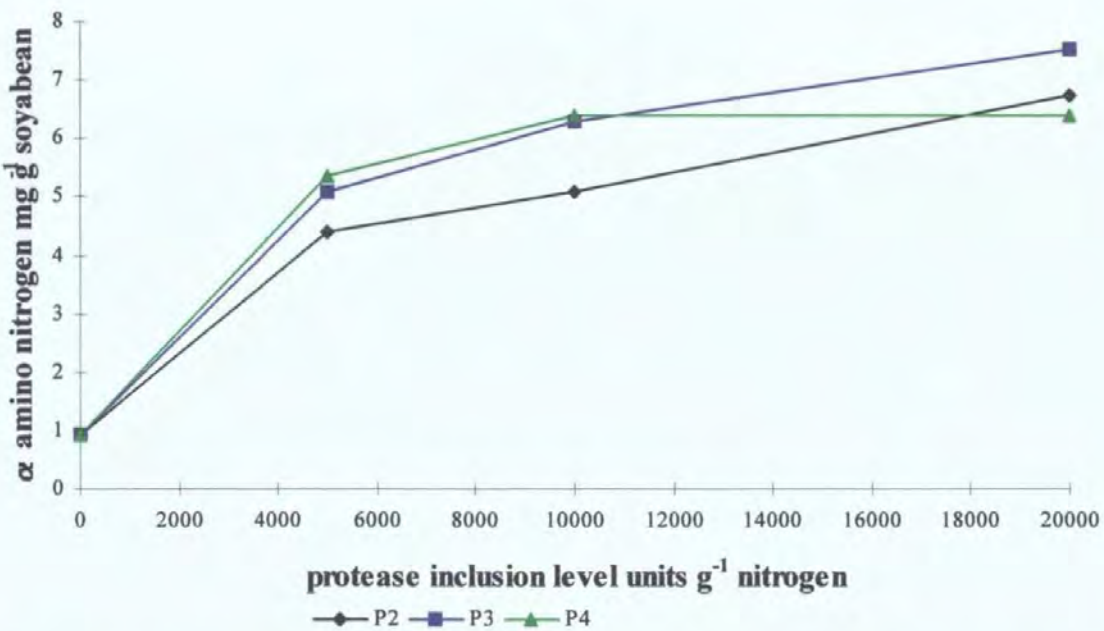


Figure 2.6.4 α amino nitrogen levels detected in soyabean slurries after steeping for 24h at 20° C with different inclusion levels of proteases P2, P3 and P4

enzyme/substrate combinations whereas ground soyabean not only contains a complex mixture of substrates but also contains other substances that may interfere with enzyme action. The ground soyabean would also contain a complex matrix of intact and fragmented cells in a dehydrated state and it is possible that a degree of hydration was necessary to allow the enzymes access to the protein. At the higher concentrations of protease the rate of hydrolysis appeared to increase rapidly after six hours steeping which suggests that if hydration was involved in delaying enzyme action it took at least 6 h for the raw soya particles to take up sufficient water for the proteases to be able to access the substrate and for activity to occur. Had samples been taken between the 6h and 18h sampling points a more conventional hyperbolic plot may have been obtained and the time taken for any appreciable enzyme activity to occur determined more accurately. Having said that, when using proteases to pretreat soya for inclusion into liquid feed for pigs, 24 h would be a convenient steeping time because the protease could be added to the soya component of the diet the day before feeding.

In this study the pH of the slurries steeped at pH 4 was adjusted to pH 6.5 prior to extraction in order to aid protein solubility. The lack of a significant difference between α-AN extracted from the control samples steeped at pH 4 and pH 6.5 indicate that by using this technique the adverse effects of pH on protein solubility were overcome.

In study 2.4. α-AN production, in the 0.25 mm particle size, increased by 1.8 mg g^{-1} soya after 24 h of steeping in the presence of 1 000 units P3 g^{-1} N. In the present study α-AN increased by 4.3, 5.4 and 6.7 mg g^{-1} soya after steeping for 24 h with inclusion levels of 5 000, 10 000 and 20 000 units P3 g^{-1} N respectively. This represents a *circa* 4-fold increase in α-AN in response to a 20-fold increase in enzyme inclusion levels. This was probably due to the rapid depletion of available scissile bonds with high enzyme inclusion

levels. α-AN increased by a further 0.58 mg g^{-1} soya after steeping for 3 d in the presence of 1 000 units P3 g^{-1} N (Study 2.4). However, the increase in protein hydrolysis observed with the high enzyme inclusion rates used in the present study indicate that maximum hydrolysis was not achieved with 1 000 units P3 g^{-1} N over longer steeping times. This supports the suggestion made in section 2.5.4 that the fall in pH observed over a steeping time of 3 d affected the action of P3 either by rendering the protein insoluble or by forcing the enzyme to work at a suboptimal pH or a combination of the two factors.

The decision to use high enzyme inclusion rates of 20 000 units g^{-1} N was made in order to achieve a maximum degree of hydrolysis in a 24 h period.

2.6.5 *Conclusions.*

A maximum degree of hydrolysis of soyabean protein, as determined by increases in α-AN, was achieved by treatment of soyabean slurries with 20 000 units P3 g^{-1} N and 10 000 units P4 g^{-1} N. Inclusion of P2 at the higher inclusion rate produced comparable increases in α-AN; however, the levels of α-AN did not reach a maximum. Soyabean contains a complex mixture of proteins which may not be equally susceptible to hydrolysis by the same enzymes. There was no information available at this time on the scissile bond specificities of the proteases nor whether they were endoproteases or exoproteases. Whilst the production of α-AN in the soyabean slurries demonstrates that protein is being hydrolysed by these enzymes, it gives no indication of which proteins are being hydrolysed or of the relative size of the resultant polypeptides. Therefore further studies are needed to determine the effect of these proteases on the proteinaceous ANF content of raw soyabean and on the storage protein.

2.7 The effect of proteases P2, P3 and P4 on the trypsin inhibitor activity of isolated Kunitz trypsin inhibitor and ground raw soyabean.

2.7.1 *Introduction.*

The main proteinaceous ANF's in raw soyabean are reputed to be the serine protease inhibitors. These are estimated to account for 40% of the reduction in growth performance observed when animals are fed diets containing raw soyabean (Liener and Kakade 1980). Schulze *et al.* (1993a) demonstrated that significant (P <0.05) dose related losses of endogenous nitrogen were incurred in pigs fed diets containing trypsin inhibitor activities greater than 2.5 mg trypsin inhibited g^{-1} diet. Rackis *et al.* (1986) comprehensively reviewed the trypsin inhibitor content of a range of oilseeds and legumes and reported the trypsin inhibitor activity of raw soyabean to range from 47 to 83 mg trypsin inhibited g^{-1} soyabean protein. If raw soyabean with crude protein content of 40% was included in a pig diet at an inclusion level of 25 % this could represent a trypsin inhibitor activity ranging between 4.7 and 8.3 mg trypsin inhibited g^{-1} diet, which, even at the lower level, is above the threshold level recommended by Schulze *et al.* (1993a). Any substantial reduction in trypsin inhibitor activity due to hydrolysis of serine protease inhibitors by exogenous enzymes could enable raw soyabeans to be included in pig diets.

The previous studies demonstrate that P2, P3 and P4 were capable of hydrolysing soyabean protein. However, they give no indication of whether the serine proteases were degraded and inactivated. Serine protease inhibitors represent approximately 6 % of the total soyabean protein, (Hymowitz 1984), and are, therefore, considerably diluted in the ground soyabean matrix. As the other soya protein will compete for protease binding sites, little enzymatic degradation of the SPI may occur.

The objectives of this study were to:-

- determine whether the proteases, P2, P3 and P4, were capable of degrading SPI's as determined by the effect of enzyme treatment on the activity of isolated KTI.
- determine the effect of pretreatment of soyabean slurries with P2, P3 and P4 on the trypsin inhibitor content of raw soyabean.

2.7.2 *Materials and methods.*

A study was conducted to investigate the effect of exogenous enzyme treatment on isolated soyabean Kunitz trypsin inhibitor (KTI) and on the trypsin inhibitory activity (TIA) of raw soyabean (RSB). The treatments used to investigate the effect of proteases P2, P3 and P4 on isolated KTI and the TIA activity of raw soyabean are presented in Table 2.7.1. All treatments were replicated three times.

Table 2.7.1. Identification codes of treatments used in study 2.7

Code	Material	pH	Protease addition units g^{-1} N
C1	KTI	6.5	0
$P3_1$	KTI	6.5	1 000
$P4_1$	KTI	6.5	1 000
C2	KTI	4	0
$P2_1$	KTI	4	1 000
C3	RSB	6.5	0
$P3_{20}$	RSB	6.5	20 000
$P4_{20}$	RSB	6.5	20 000
C4	RSB	4	0
$P2_{20}$	RSB	4	20 000

KTI: isolated soyabean Kunitz trypsin inhibitor 2 mg ml^{-1}
RSB: ground raw soyabean slurry, water : soya = 3 : 1

The enzyme concentrations were calculated to provide an enzyme : trypsin inhibitor ratio equivalent to that present in ground raw soyabean treated with 20 000 units protease g^{-1} N, assuming a trypsin inhibitor content of approximately 6%. A solution of 2 mg ml^{-1} isolated soyabean trypsin inhibitor (Sigma T9128) was prepared and dispensed into 1ml aliquots. The pH of this solution was pH 6.5. A further solution of the same concentration was prepared and adjusted to pH 4 with 4M HCl. Ground raw soyabeans slurries were prepared according to the method described in section 2.4.3. Samples of KTI and raw soyabean slurries were treated according to the treatments described in Table 2.7.1 and all samples were incubated at 20° C for 24h. At the end of the incubation period the raw soya slurries were homogenized in a blender (Commercial Bar Blendor, Waring USA). Approximately 1 ml of each homogenate was accurately weighed (± 0.01g) and transferred to a 100 ml flask and 50 ml 0.01 M NaOH added and the trypsin inhibitor extracted according to the method of Smith, van Megen, Twaalfhaven and Hitchcock (1980). Subsamples of 0.25 ml of the isolated KTI samples were taken at the start of the incubation period and after 24 h. Trypsin inhibitor activity of the extracted raw soya samples, a sample of the dry ground RSB and the isolated KTI samples was determined according to the method of Smith *et al.* (1980)(Appendix 6). Three replicate analyses were conducted for each subsample.

The results were analysed by single factor analysis of variance using Minitab (Minitab inc. release 10.2, State College, Pennsylvania, 1994). Treatment means were compared using Newman- Keuls multiple range test (Zar 1999).

2.7.3. *Results.*

The method of Smith *et al.* (1980) determines trypsin inhibitor activity by measuring residual trypsin activity with the synthetic chromogen, benzoyl-arginine-p-nitroanilide (BAPNA). It was notable that in this study all three of the proteases interfered with the

assay, having some apparent trypsin inhibitory activity. When the proteases alone were included in the assay mixture they all produced trypsin inhibitory activities of 0.3 mg trypsin inhibited per sample. Two possible mechanisms may be involved in this. Firstly the proteases may be capable of hydrolysing trypsin under the conditions of the assay. Secondly the proteases themselves may have some trypsin inhibitory activity. The assay was conducted under alkaline conditions (pH 8.2) at 37° C, therefore whilst the former explanation may be valid for P3 and P4 which have approximately neutral pH optima it is unlikely to be true for P2 which has an acid pH optimum. Therefore, P2 may have been acting as a true inhibitor.

Table 2.7.2. Trypsin inhibitor activity (mg trypsin inhibited mg^{-1} KTI) of isolated KTI treated with 0 or 1 000 units g^{-1} N of P2, P3 or P4

pH	6.5			4		
Protease	0	P3	P4	0	P2	s.e.d.
Time (h)						
0	1.1^{a1}	1.0^{a}	1.0^{a}	0.9^{1}	1.1^{a1}	0.06
24	1.1^{a1}	0.8^{b}	0.9^{b}	1.0^{a1}	1.0^{a1}	

^{a,b} means in the same row with the same superscript are not significantly different ($P > 0.05$)
^{1} means in the same column with the same superscript are not significantly different ($P > 0.05$)
All values are corrected as appropriate to allow for the apparent trypsin inhibitory activity of P2, P3 and P4.
Inclusion rate of P2, P4 and P4 = 1 000 units g^{-1} N.

The results of the trypsin inhibitor assays on isolated KTI are presented in Table 2.7.2. The data in Table 2.7.2. show that pH appeared to have a significant effect on trypsin inhibitor in the control samples before incubation. It was noticed that the trypsin inhibitor did precipitate out as pH was reduced in all of the pH 4 samples and this may have reduced the amount of trypsin inhibitor carried into some of the subsamples. After 24 h incubation at 20° C the precipitate appeared to have re-dissolved. Therefore, the apparent ability of P2 to increase trypsin inhibitor activity in the presence of KTI should be interpreted with caution.

Also the possibility of an interaction between KTI, P2, trypsin and BAPNA cannot be discounted. The data obtained for the remaining samples at 0 h and for the control samples at 24 h are in agreement with the stiochiometrically equivalent relationship generally reported for the KTI/trypsin complex.

The results for the trypsin inhibitor activity of ground raw soyabean are presented in Table 2.7.3. There was a significant reduction in trypsin inhibitor activity due to protease treatment in all cases but the differences between proteases were not significant. Again these data needed to be interpreted with caution because the addition of the proteases themselves had an effect on the assay, producing mean trypsin inhibitor activities of 6.4, 6.3 and 6.6 mg trypsin inhibited per sample for P2, P3 and P4 respectively.

Table 2.7.3. Trypsin inhibitor activity (TIA) of raw soyabean slurries steeped for 24 h at 20° C with 0 or 20 000 units g^{-1} N of P2, P3 or P4.

pH	6.5				4		
Steeping time (h)	0	24			24		
Protease	Control	Control	P3	P4	Control	P2	s.e.d.
TIA (mg trypsin inhibited g^{-1} soyabean DM)	28.5[a]	27.6[a]	17.2[b]	18.5[b]	25.7[a]	20.0[b]	1.60

[a,b] means are not significantly different ($P > 0.05$)
All values are corrected as appropriate to allow for the apparent trypsin inhibitory activity of P2, P3 and P4.
Inclusion rate of P2, P3 and P4 = 20 000 units g^{-1} N

The data obtained from the original ground soyabean and from the control samples, when translated into mg trypsin inhibited g^{-1} protein, gave mean values of 71.3, 67.0 and 64.3 (s.e.d. 5.50) for dry soyabean and the samples steeped in the absence of protease at pH 6.5

and pH 4 respectively. These values were in accordance with those published by Rackis *et al.* (1986).

2.7.4 *Discussion.*

The first part of this study demonstrated that P3 and P4 were capable of reducing the activity of isolated KTI at enzyme inclusion ratios likely to prevail in ground raw beans at this level of enzyme inclusion, which suggests the differences observed in Table 2.7.3 reflect a true reduction in trypsin inhibitor content. The data for P2 treatment are more difficult to interpret, as the results obtained in the first part of this study indicate that P2 is not capable of hydrolysing KTI in isolation. However, the trypsin inhibitor activity in raw soyabean is provided by both KTI and the Bowman-Burke trypsin/chymotrypsin inhibitor (BBI); therefore P2 could have had an effect on BBI activity.

The assay measures trypsin inhibitor activity by comparing the amount of p-nitroanilide conjugate released by cleavage of BAPNA in the trypsin standard to that released in the sample. Therefore, as Smith *et al.* (1980) point out, the presence of protein other than trypsin inhibitors in the samples could affect the outcome of the assay by providing substrates that compete with BAPNA for trypsin binding sites. This would result in a reduction in the release of p-nitroanilide which would give a false impression of trypsin inhibitory activity. In this respect the hydrolysis products of the proteases may be more competitive than intact soyabean protein. Therefore, trypsin inhibitor activity in the protease treated soyabean may actually be lower than this assay indicated.

Recent work by Vaintraub and Haram (1995) on the proteolytic degradation of purified KTI demonstrated that a considerable degree of hydrolysis was required before there was a significant reduction in inhibitory activity. Vaintraub and Haram (1995) used papain,

subtilisin or pepsin in their study and discovered that partial hydrolysis of the KTI protein molecule had to proceed to the stage at which 80% of it was degraded to small, trichloracetic acid (TCA) soluble peptides for the activity to be reduced to 20% of its initial value (initial activity unstated). This suggests that the integrity of the active site is not disrupted during limited proteolysis. In this study 69% and 78% of the initial activity of KTI remained after treatment with P3 and P4 respectively.

The threshold level of trypsin inhibitor activity (TIA) for inclusion into pig diets postulated by Schulze *et al.* (1993a) was 2.5 mg trypsin inhibited g^{-1} diet. If the soyabean meal content of a typical pig diet is taken to be 25 % then replacing processed soyabean meal with raw soya would provide 6.9 mg trypsin inhibited g^{-1} diet. By pretreating the raw soya with P2, P3 or P4 the TIA would be reduced to 5.0, 4.6 or 4.3 mg trypsin inhibited g^{-1} diet respectively. Therefore, although all protease treatments reduced trypsin inhibitor activity in raw soyabean slurries, the reduction would not be sufficient to bring the level of trypsin inhibitory activity below the threshold level postulated by Schulze *et al.* (1993a).

Alternatively, using Schulze *et al.*'s (1993a) value of 2.49 mg trypsin inhibited g^{-1} diet, the maximum quantity of raw soyabean with a TIA of 28.5 mg trypsin inhibited g^{-1} RSB that could be used in a pig diet would be 8.7 %. This quantity could be increased to 12.5 %, 14.5 % or 13.5 % if RSB was pretreated with P2, P3 or P4 respectively.

These studies indicate that treatment of soyabean slurries with protease P3 and P4 and possibly with P2 could be useful in reducing trypsin inhibitor levels in raw soyabean, but would be unlikely to render raw soyabean suitable for use in pig diets at levels greater than 12 - 14 %.

2.8 An assessment of the use of gel electrophoresis techniques to determine the qualitative effect of proteases P2, P3 and P4 on soyabean protein.

2.8.1. *Introduction.*

The studies reported thus far demonstrated that all three proteases were capable of hydrolysing soyabean protein at inclusion levels of 20 000 units g^{-1} N. Although the production of αAN shows that hydrolysis is taking place it gives no indication of the effect of the proteases on individual soyabean proteins.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) is a technique widely used to separate proteins according to their molecular weights (MW). In this technique proteins are denatured by boiling with a detergent, SDS, which disrupts the quaternary and tertiary structure of the protein and imparts a constant charge : weight ratio. A thiol-reducing agent is generally added during the denaturation process to cleave disulphide bonds. The resultant negatively-charged denatured proteins migrate through polyacrylamide gel upon application of an electric field and fractionate into discrete bands according to their MW.

The technique of SDS PAGE has been successfully used by a number of researchers to assess the effect of enzymatic digestion of soyabean protein. Nielsen *et al.* (1988) and Ikeda *et al.* (1995) used the technique to determine the digestibility of legume storage proteins and soya protein foods respectively. Romagnolo, Polan and Barbeau (1990) assessed the rumen degradability of soyabean protein fractions using SDS PAGE. Rooke *et al.* (1998) reported different patterns of hydrolysis for two exogenous proteases as revealed by SDS PAGE.

Ninety percent of the protein in soyabeans consists of the globular storage proteins, glycinin and β-conglycinin. Each is a multimeric protein consisting of a variable number of subunits. The molecular mass of glycinin ranges between 310 kDa and 350 kDa and that of β-conglycinin between 150 kDa and 200kDa (Nielsen 1983). Under the denaturing and reducing conditions of SDS PAGE sample preparation, β-conglycinin separates into three subunits, α, α_1 and β of apparent MW 83 kDa, 72 kDa and 48 kDa respectively, whereas glycinin separates into acidic and basic subunits of apparent MW 40 kDa and 20 kDa respectively (Romagnolo *et al.* 1990; Tukur, Lalles, Mathis, Caugant and Toullec 1993). However, Nielsen (1985) points out that the separation of glycinin into acidic and basic subunits is largely an artefact of the sample preparation and that glycinin exists as a hexamer, each subunit (MW ~ 60 kDa) of which has an acidic and a basic component held together by disulphide bridges, and it is these components which have been erroneously regarded as glycinin subunits. In respect to this study, it is the intact protein that represents the substrate for exogenous enzymatic degradation. In the case of glycinin, disulphide bonds hold the acidic and basic components of the subunits together, and it is the intact subunit which forms the substrate for the enzyme. Therefore, sample preparation techniques which disrupt the composition of the protein may give a false impression of the action of the enzyme in terms of which proteins it degrades. For example, if the result of hydrolysis by any of the exogenous proteases is cleavage of peptide components either side of the disulphide bond, and these bonds are subsequently disrupted by sample preparation, then the resultant peptide banding pattern may erroneously indicate the formation of low MW peptides. However, omitting the reducing agents from sample preparation can lead to difficulty in interpreting protein banding patterns if disulphide bridges play a major role in maintaining tertiary structure. This is because non-reduced peptides may have a more compact shape than their reduced counterparts and will migrate to different positions in the gel (Hames 1990). In the case of glycinin there is some evidence that intrapeptide

disulphide bonds may maintain the tertiary structure of the acidic component, (Nielsen 1985). However, the tertiary structure of basic glycinin components and of β-conglycinin subunits is maintained primarily by hydrophobic groups (Kinsella, Damodaran and German 1985). Therefore, it was felt that SDS Page run under non-reducing conditions may be appropriate in this study. The objectives of this study were to determine the usefulness of conducting SDS PAGE under non-reducing conditions in evaluating the effect of P2, P3 and P4.

2.8.2 *Materials and methods.*

Ground raw soyabean slurries were prepared according to the method in section 2.4.3. and received the treatments described in Table 2.8.1.

Table 2.8.1 Identification codes and treatments of ground raw soyabean slurries used in study 2.8.

Code	pH	Protease treatment (units g^{-1} N)
C1	6.5	0
$P3_{20}$	6.5	20 000
$P4_{20}$	6.5	20 000
C2	4	0
$P2_{20}$	4	20 000

Slurries were steeped for 24 h at 20° C. Subsamples, taken initially and after steeping for 24 h, were homogenized in a blender. Immediately after homogenization duplicate, 0.25 g, samples of each homogenate were mixed with 1 ml hot buffered SDS (0.5M tris pH 6.8, 2 % SDS, 0.002 % bromophenol blue, 5 % glycerol) with or without 2-mercaptoethanol (5 %) and boiled for 3 min. Five µl of each sample was loaded into wells in a polyacrylamide slab gel (4 % stacking gel, 12.5 % resolving gel) and electrophoresis was

conducted at 100 mv using a discontinuous buffer system (Hames 1990) in a minigel system (Biorad miniprotean II). The resolving gel buffer and electrode buffer consisted of 1.5M tris pH 8.8 and 0.25M tris, 1.92M glycine, 1 % SDS pH 8.3 respectively.

After electrophoresis gels were stained with Coomassie blue (0.1% in 50% methanol/ 10 % acetic acid) and photographed prior to drying using a gel dryer (Biorad). Densitometry (Ultroscan XL, LKB, Bromma, Sweden) was conducted on suitable gels containing non-reduced samples.

2.8.3 *Results and discussion.*

An electrophoresis gel comparing the effect of denaturation of soyabean protein under reducing and non-reducing conditions is presented in Figure 2.8.1. This shows the redistribution of protein bands in the unhydrolysed soya due to thiol treatment during sample preparation. In the reduced soya, major groups of bands fractionate at apparent MW of 72 kDa, 62 - 67 kDa, 57 kDa, 36 kDa and 34 kDa and 18 - 20 kDa. In the non-reduced soya main group of bands fractionate at MW's of 63- 88 kDa and 48 -52 kDa bands also appear at MW's of ~ 42 kDa, ~ 30 kDa and ~ 18 kDa. A tentative identification of the subunits of glycinin and β-conglycinin and of KTI was made and these are indicated in Figure 2.8.1. In view of the disruptive effect mercaptoethanol had on the larger MW soya protein it was decided it would be desirable to omit reducing agents from the sample preparations in which the effect of exogenous proteases were being assessed.

Figure 2.8.2. shows the hydrolysis patterns for proteases P2, P3 and P4 compared with unhydrolysed raw soya protein. Densitometry was conducted on the gel containing these samples in an attempt to semi-quantify the differences between the proteases. The area

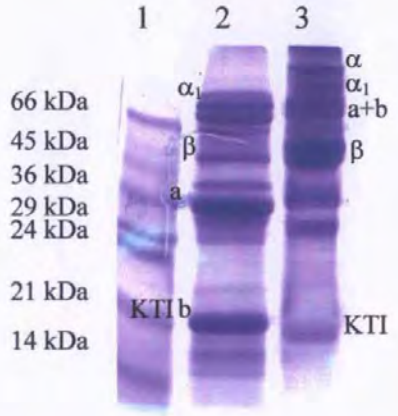


Figure 2.8.1. Electrophoresis gel of raw soyabean denatured under reducing conditions (lane 2) or non reducing conditions (lane 3). Lane 1: molecular weight standard.

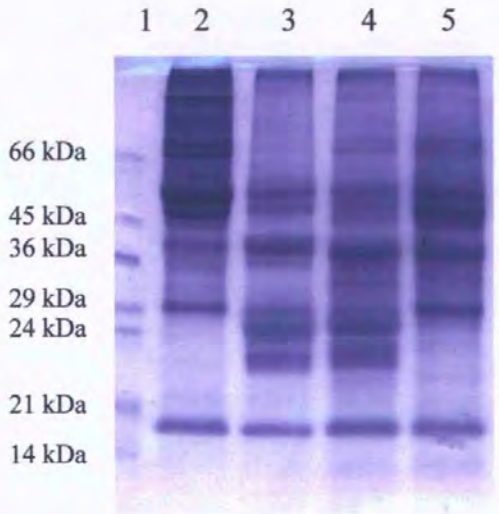


Figure 2.8.2. Electrophoresis gel of raw soyabean steeped for 24 h at 20° C in the presence of 0 (lane 2) or 20 000 units g^{-1} N of P2 (lane 3), P3 (lane 4) of P4 (lane 5). Lane 1: molecular weight standards.

under the peaks obtained from the densitometry traces, which correspond to the main protein bands, are presented in Table 2.8.2. Peptides smaller than about 10 kDa are not retained by polyacrylamide gels, therefore any hydrolysis products with MW's smaller than 10 kDa cannot be identified by this method.

Table 2.8.2. Apparent molecular weight and area under peaks from the densitometry trace of main protein bands in raw soyabean meal steeped for 24 h at 20° C with 0 or 20 000 units g^{-1} N of P2, P3 or P4

	Protease treatment (20 000 units g^{-1} N)			
MW band	0	P2	P3	P4
80 +	1.67			
63 - 66	1.70		0.34	0.97
48 - 52	3.05	1.44	0.95	2.35
39 - 42	1.11	1.24	1.38	1.75
29 - 30	0.88			0.92
24 -27		2.09	0.85	
18 - 20	0.37	0.32	0.28	0.48

Exact quantification of the relative amounts of protein in the MW bands was not possible as there was not sufficient separation between the bands to give distinct peaks. This was due to shortcomings of the apparatus itself, as the optics were not sufficiently sophisticated to pick up sharp bands. The densitometer measured differences in absorbance of the protein/ Coomassie blue complexes. However, different proteins bind Coomassie blue (Hames 1990) to different extents and consequently differences in density may have been due to differences in the binding efficiency of the dye rather than in quantity of protein. The data presented in Table 2.8.2. show relative differences in the pattern of hydrolysis

between the three proteases. For reasons stated previously these data needed to be interpreted with caution and it was felt that comparisons were only relevant for indicating differences between samples run on the same gel as these have been subjected to the same conditions during electrophoresis, staining and destaining. The molecular weight values stated in Table 2.8.2. are approximate as they were calculated from the motility of a range of standard proteins of known MW and the peak profile of the standard was not well defined. This was probably due to optical constraints of the instrumentation. Having said that, it was felt that some broad distinctions between the three proteases could be elucidated by this technique.

All three proteases appeared capable of hydrolysing the large MW (> 72 kDa) α and α_1 subunits of β-conglycinin. However, none of the proteases completely hydrolysed the protein in the MW band 48 - 52 kDa which contains the β subunit of β-conglycinin. With P2 and P3 diffuse bands of proteins with MW ranging from approximately 24 to 27 kDa appeared which were probably products of hydrolysis of larger MW proteins. There was a persistent band of protein of MW 18 -20 kDa in all samples. This could have been KTI and / or the basic subunit of glycinin. P2 appeared to be capable of hydrolysing proteins in the 63 - 66 kDa range whereas some of these proteins persisted in the slurries treated with P3 and P4. Rooke *et al.* (1998) reported a specific reduction in staining intensity of high molecular weight bands of processed soyabean meal due to partial hydrolysis by an acid protease, and a non-specific reduction in staining density due to partial hydrolysis by an alkaline protease. Both of these proteases were also supplied by Finnfeeds and information from the suppliers indicated that the alkaline protease used by Rooke *et al.* (1998) was identical to P3. It can only be speculated that the acid protease was the same as P2. However, it is difficult to draw direct comparisons between this study and that of Rooke *et al.* (1998) because their samples were prepared under reducing conditions, they used

processed soyabean meal and the pretreatment regime used for the soya was different (50° C for three hours).

The different patterns of hydrolysis observed in Figure 2.8.2. and Table 2.8.2. reflect differences in bond specificity and hydrolysis products between the three proteases. In respect to protein utilization by the pig the implications of partial hydrolysis of soya protein are uncertain. β-conglycinin has often been implicated in causing allergic reactions in pre-ruminant calves and young pigs (Lalles *et al.* 1993; Tukur *et al.* 1993). Therefore, partial hydrolysis of these components of soya protein may have some benefit.

2.9. Conclusions

Little work has been reported on the use of protease pretreated soyabean meals in pig diets. Caine *et al.* (1997a), Hessing *et al.* (1996) and Rooke *et al.* (1998) have reported the use of an alkaline protease and an acid protease in the pretreatment of conventional soyabean meal in diets for newly weaned pigs. The proteases used by these authors were also supplied by Finnfeeds and the alkaline protease they used was the same as P3 used in the present study (H. Schulze, Finnfeeds International Ltd. Marlborough, personal communication). The studies reported in this chapter demonstrate that all three exogenous proteases were capable of hydrolysing raw soyabean protein in soyabean slurries steeped at 20° C. The inclusion levels of 20 000 units g^{-1} N effectively translate to 0.25 %, 1 % and 0.5 % for P2, P3 and P4 respectively. In comparison, inclusion levels of proteases used by Caine *et al.* (1997a), Hessing *et al.* (1996) and Rooke *et al.* (1998) were 0.1 % regardless of activity. Of these authors Rooke *et al.* (1998) reported increases in α amino nitrogen production in soyabean meal of 44 g kg^{-1} N and 49 g kg^{-1} N due to pretreatment at 50° C for 3 h with an alkaline and an acid protease respectively compared to alkali or acid treated controls. Although it is not clear from the study of Rooke *et al.* (1998) what type of soyabean meal the authors used in their study, if it is assumed that it was a conventional solvent extracted soyabean meal then these figures equate to increases of 3.9 and 3.5 mg αAN g^{-1} soyabean meal. These values are lower than those achieved in the present study where inclusion levels of 20 000 units g^{-1} N gave increases of 5.3, 6.3 and 5.2 mg g^{-1} RSB with respect to untreated soyabean slurries steeped for 24 h for P2, P3 and P4 respectively. This may reflect either the lower inclusion level of the proteases used in the study of Rooke *et al.*. (1998) or the ability of the proteases they used to hydrolyse processed soyabean meal. The authors did not report whether they had used αAN production to optimize enzyme inclusion levels.

The higher inclusion levels used in the present study were selected to achieve the optimum protein hydrolysis at 20° C and reflect the fact that the proteases were probably operating at sub-optimal temperature. The temperature constraint was imposed as ultimately one of the objectives of this project was to develop an on farm regime for pretreating soyabean meal for inclusion into liquid feed for pigs. Therefore, with this in mind the suitability of protease P2 must be questioned. Although P2 is effective in hydrolysing soyabean protein its optimum pH is pH 4 and its use would necessitate reducing the pH of the soyabean slurries with an acid. This has safety implications in respect of the storage and handling of hazardous substances. In these laboratory studies HCl was used to reduce the pH of the slurries to be treated with P2. However, Roth and Kirchgessner (1998) reported that HCl reduced feed intake in young pigs. In theory organic acids could be used to reduce the pH, but these are expensive and their use would still have implications regarding the safe storage and handling of hazardous substances.

The reduction in trypsin inhibitor activity due to protease pretreatment may be reflected in improved utilization of raw soyabean for pigs. However, as trypsin inhibitor activity was still present above the threshold level postulated by Schulze *et al.* (1993a) it is unlikely that protease treated raw soyabean would achieve the same nutritional status as processed soyabean meal, although it could increase the level at which raw soyabean could be introduced into the diet before presenting problems of increased endogenous nitrogen loss.

At this stage of the project the relevance of the partial hydrolysis of raw soyabean to improvement of protein utilization in the pig was uncertain. β-conglycinin has been reported to cause allergenic reactions in young pigs (Lalles *et al.* 1993; Lalles and Salmon 1994), and it could be speculated that partial hydrolysis of this protein may have some beneficial effects on growth performance of the pig. Predigestion of some of the large

molecular weight proteins may also aid protein digestibility by presenting smaller polypeptides to digestive proteases or possibly by opening up the globular structure of the protein and thereby facilitating access for digestive enzymes.

Chapter 3

The effect of protease pretreatment of raw and differently processed soyabean meals on α-amino nitrogen production, electrophoresis profiles and *in vitro* nitrogen digestibility.

3.1 Introduction

In the studies reported in chapter 2 a regime was developed for the pretreatment of raw soyabean with exogenous proteases that could be used in an 'on farm' process for use in liquid feed systems for pigs. α-AN production and SDS PAGE profiles were used to obtain quantitative and qualitative estimates respectively of the effect of different proteases on raw soya protein. Raw soyabean is not used in pig diets due to the detrimental effect on growth performance attributed to the presence of ANF's; however processed soyabean meals are used ubiquitously. It was considered that pretreatment of processed soyabean meals with exogenous enzymes may give some benefit in increasing protein digestibility due to partial hydrolysis of large MW proteins prior to ingestion by the pig. In the studies reported in this chapter the techniques described in chapter 2 were extended to determine the effect of exogenous protease pretreatment on processed soyabean meals.

The properties of proteins are changed during heat processing due to heat denaturation (Kinsella *et al.* 1985). During heat denaturation the tertiary and quaternary structure of protein is disrupted and this results in the loss of functionality of biologically active proteins such as enzymes. In the case of soya protein heat denaturation also inactivates ANF's such as serine protease inhibitors and lectins. One of the main effects of heat denaturation is a reduction in protein solubility. This occurs at moist (steam) processing temperatures below 140° C, above which solubility increases due to dissociation and

degradation of polypeptides (Wolf 1978) which is generally associated with protein damage and loss of nutritional quality (Melcion and van der Poel 1993). Denaturation of soya protein is achieved at lower temperatures (90 - 100° C) under moist conditions (steam processing) than with dry heat processes which require higher temperatures (120 - 190° C). The processing conditions may affect soya protein as there is evidence that, as denaturation progresses, subunits of glycinin and β-conglycinin form aggregates and macro-complexes under certain conditions of temperature and moisture (Damodaran and Kinsella 1982; German, Damodaran and Kinsella 1982; Utsumi, Damodaran and Kinsella 1984). Therefore, it is possible that soyabean meals from different processes may vary in their susceptibility to hydrolysis by P2, P3 and P4. In the following studies the effect of protease pretreatment of a range of processed full fat soyabean meals was investigated. The soyabean meals used came from different sources. Different soyabean cultivars and the climatic conditions at the time of cultivation can affect the relative proportions of glycinin and β-conglycinin in the seed (Nielsen 1983). This may have an affect on the degree of hydrolysis with the different SBM/protease combinations if there is a large variation in the amount of β-conglycin between the SBM's. However, processing treatments alter the properties of the proteins, which may result in different hydrolysis products between the different SBM/protease combinations.

3.2 Determination of an adequate heat treatment for raw soyabeans.

3.2.1. *Introduction.*

With a future feeding trial in mind, in which the growth response of pigs to protease pretreated raw and processed soyabean would be investigated, it was considered desirable that processed soyabeans and raw soyabeans from the same batch were used. In order to achieve this, it was decided to process the soyabeans on site by autoclaving. The quality of soyabean meals is assessed commercially by measuring residual urease activity, protein dispersibility and trypsin inhibitor activity (Wright 1981). The apparatus required to determine the protein dispersibility index of soyabean meal was not available, therefore urease activity and trypsin inhibitor activity were used to assess the adequacy of autoclave treatments. The degree of protein denaturation during heat processing is dependant on the time, temperature and moisture conditions under which processing occurs. The objective of this study was to determine the conditions of time and temperature of autoclaving required to reduce trypsin inhibitor activity to an acceptable level.

3.2.2 *Materials and methods.*

Autoclaved soyabeans were prepared as follows: whole raw soyabeans (obtained from Parnutt Foods, Sleaford, Lincolnshire, UK) were spread onto metal trays to a depth of 2cm. These were subjected to three different treatments, in a laboratory autoclave, of 109° C (5 lb in^{-2}) for 5mins, 115° C (10 lb in^{-2}) for 5mins and 121° C (15 lb in^{-2}) for 5mins. The time was measured from the point at which the appropriate temperature was reached and at the end of each time period the pressure was released immediately. The soyabeans were removed from the autoclave and allowed to cool to room temperature in a desiccator. Each sample of soya beans was ground in a hammer mill using a 1.5mm screen and sieved through a 0.5 mm sieve to obtain samples with a particle size of <0.5 mm. The adequacy

of heat treatments was determined by measuring the Urease index according to the official method of the A.O.C.S (1988), and trypsin inhibitor activity according to the method of Smith *et al.* (1980).

3.2.3 *Results.*

Urease activity and trypsin inhibitor activity for the autoclaved soyabean samples are presented in Table 3.2.1. Urease index values of 0.2 - 0.05 pH unit indicate that processed soyabean meals have received adequate heat treatment (Wright 1981). The Urease index values for all autoclave treatments were within this range.

Trypsin inhibitor activity was significantly reduced (P <0.001) by all autoclave treatments compared to raw beans. Trypsin inhibitor activity was also significantly reduced (P <0.05) in the samples autoclaved at 115° C and 121° C compared to the sample autoclaved at 109° C.

Table 3.2.1 Urease activity and trypsin inhibitor activity in raw and autoclaved soyabean meal

Treatment	Urease index (pH units)	Trypsin inhibitor activity (mg trypsin inhibited g^{-1} SBM (DM))
Raw	2.05	24.66
109° C / 5 min	0.07	1.42
115 ° C / 5 min	0.06[a]	0.04[a]
121° C / 5 min	0.05[a]	0.04[a]
s.e.d.	0.006	0.51

[a]means in the same column are not significantly different

3.2.4 *Discussion and conclusions.*

The trypsin inhibitor activity values for the autoclaved beans were rather low compared with data published in other studies. Herkelman *et al.* (1992) and Saini (1989) obtained TIA values of 4.8 mg trypsin inhibited g^{-1} soyabeans autoclaved at 110° C for 20 min and 1.7 mg trypsin inhibited g^{-1} soyabeans autoclaved at 121° C for 5 min. respectively. Liener and Tomlinson (1981), on the other hand, recorded trypsin inhibitor activities of < 1 μmol trypsin inhibited g^{-1} protein after autoclaving untoasted soya flour at 121° C for 2 min. These results are somewhat variable. However, all authors were measuring low levels of trypsin inhibitors in samples with a high protein content (*circa* 40 %). Smith *et al.* (1980) discussed the difficulty of obtaining reliable results from such samples, as the presence of protein in high concentrations interfers with the assay by competing with BAPNA for trypsin binding sites. Leiner and Tomlinson (1981) stated that 79 - 87% of trypsin inhibitor needed to be destroyed to obtain maximum weight gains and protein digestibility in rats. In the present study trypsin inhibitor activity was reduced by 94 % for beans autoclaved at 109° C and 99 % for beans autoclaved at 115° C and 121° C. The results of both the urease index and the trypsin inhibitor assay indicated that the soyabeans autoclaved at 109° C for 5 min had received adequate heat treatment. Therefore in future studies this heating regime was used to prepare autoclaved soyabean meal.

3.3 The effect of exogenous protease pretreatment on protein hydrolysis of raw soyabean and four processed soyabean meals as determined by α-amino nitrogen production and SDS PAGE.

3.3.1 *Introduction.*

The studies reported in Chapter 2 demonstrated that the proteases P2, P3 and P4 were all capable of hydrolysing raw soya protein to varying degrees. The measurement of α-AN production can be used to quantify the degree of hydrolysis due to protease treatment, whilst SDS PAGE provides a useful qualitative technique to visualize the relative degradation of the different proteins and their hydrolysis products. Immunoblotting is a technique whereby proteins separated by SDS PAGE are transferred onto a solid medium and probed with specific antibodies. As the antibodies bind to their specific epitopes they indicate the location of specific proteins within the SDS PAGE profile. Providing the epitopes are not destroyed by hydrolysis this technique may provide useful information as to the degree of degradation of specific soya proteins, if their positions in the profile change with respect to unhydrolysed controls.

The objectives of this study were to determine:

- the effect of P2, P3 and P4 on a range of processed soyabean meals by measuring α-AN production.
- any differences in hydrolysis products by SDS PAGE profiles and immunoblotting techniques.

3.3.2. *Materials and methods.*

A study was conducted using a 4 x 5 factorial design to examine the effect of three protease treatments on α-AN production and SDS PAGE profiles in raw soya and four soyabean meals produced using different processing methods.

Two commercially processed soyabean meals were obtained from Primary Diets (Ripon, Yorkshire, UK). Steam pressured cooked (SPC) beans had been steam pressure cooked at a temperature of 110° C to 120° C for a dwell time of 15 -20 minutes followed by drying with hot air and cooling prior to rolling and milling. Micronized beans (MIC) were steam conditioned prior to micronization, rolling and milling. A further product was obtained from a local feedmill (ISCA agriculture, Exeter, UK). The only information on the processing of this product was that it was toasted (TSD). Autoclaved soyabean meal (AUT) was prepared in the laboratory according to the process reported in section 3.2. All soyabean meals were sieved through a 0.5 mm sieve prior to the following investigations.

Slurries were prepared from the four processed soyabean meals, SPC, MIC, TSD and AUT and raw soyabean meal (RSB) and three replicate samples of each were pretreated with either 0 or 20 000 units of P2, P3 or P4 g^{-1} N. The slurries treated with P2 were adjusted to pH 4 with 4M HCl. Control samples at pH 4 were not included in this study because previous studies showed that there was no significant difference in α-AN extraction between untreated soyabean meal steeped at pH 4 and pH 6.5 provided the pH of the extraction buffer was adjusted to pH 6.5. All samples were incubated for 24 h at 20° C. Samples of each meal prior to pretreatment, and of each slurry after incubation were taken for α-AN determination and SDS PAGE and processed according to the methods described in chapter 2 sections 2.6.2 and 2.8.2 respectively. Suitable gels were scanned on a densitometer and the apparent molecular weights of the main peaks were determined.

Several pairs of SDS PAGE gels were run and the protein from one gel of each pair was transferred onto nitro-cellulose paper using the Western blot technique. Transfer was conducted overnight in a transblot cell (Scotlab) at 30 mV with a transfer buffer (0.7 % ethanoic acid, 20 % methanol in deionized water) (Hames 1990). Separate nitro-cellulose blots were probed with the following antibodies, anti-KTI (mouse, monoclonal), anti-soyabean lectin (mouse, monoclonal), anti-11S glycinin (mouse, monoclonal) and anti-11S glycinin (rabbit, polyclonal), all supplied by J. Thorpe, University of Bristol Veterinary school, or rabbit anti-soy protein (Sigma). Bound antibodies were detected using an immunoblot kit (Biorad) with anti-mouse or anti-rabbit horseradish peroxidase (HRP) conjugates, stained with an HRP chromogenic substrate according to the instructions provided by the supplier.

The results of the α-AN assays were analysed statistically by analysis of covariance with the α-AN levels prior to steeping as the covariate factor. All data were checked for normality prior to statistical analyses using Minitab (Minitab inc. release 10.2, State College, Pennsylvania, 1994) and treatment means were compared using Newman - Keuls multiple range test.

3.3.3. *Results.*

The mean levels of α-AN extracted from the SBM samples before steeping were 0.54, 0.29, 0.32, 0.24 and 0.29 for RSB, SPC, MIC, TSD and AUT respectively. These values were used as covariates in the statistical analyses. The factorial analysis of the adjusted means for the production of α-AN in response to treatment with proteases P2, P3 and P4 in raw soyabean and the four differently processed soyabean meals, SDC, MIC, TSD and AUT, is presented in Table 3.3.1. The data presented in this Table show that overall α-AN production was greatest with P3 which increased α-AN production by 5.96 mg g^{-1} soya

with respect to no protease treatment compared with increases of 4.54 and 5.16 (s.e.d. 0.06) mg g^{-1} soya for P2 and P4 respectively. The interaction between SBM and protease was highly significant ($P < 0.001$) with all protease pretreatments increasing α-AN production in all soyabean meals. However, when individual means were compared, no single protease showed a greater increase in α-AN production in all soyabean meals.

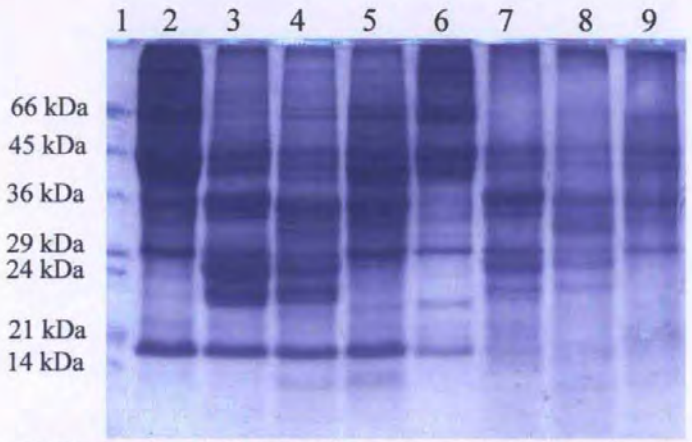
Table 3.3.1. Adjusted means of α-amino nitrogen (mg g^{-1} soyabean meal) production in raw (RSB), steam pressure cooked (SPC), micronized (MIC), toasted (TSD) and autoclaved (AUT) soyabean meals in response to pretreatment with 0 or 20 000 units g^{-1} N of P2, P3 or P4.

SBM	Protease				Main effect: SBM	s.e.d.
	0	P2	P3	P4		
RSB	0.80	6.02[1]	7.88	7.38	5.52	0.030
SPC	0.29[1]	6.02[1]	5.25[1]	4.82	4.10[1]	
MIC	0.32[1]	5.43	5.89	4.64	4.07[1]	
TSD	0.26[1]	3.30	5.38[1]	4.43	3.34	
AUT	0.31[1]	3.87	7.34	6.49	4.50	
Main effect: protease	0.39	4.93	6.35	5.55		
s.e.d.	0.030					

s.e.d. for the interaction between SBM and Protease = 0.06
[1] means in the same column are not significantly different.

The electrophoresis gels for the various SBM /protease combinations are presented in Figure 3.3.1. - 3.3.3. The gels showed that slightly different hydrolysis patterns were obtained for different SBM / protease combinations. The apparent molecular weights determined from densitometry traces of the gels and the area under each peak, which represents the density of staining of the respective protein bands, are presented in Tables 3.3.2, 3.3.3 and 3.3.4. All SBM's showed a reduction in the size and staining density of higher molecular weight proteins due to all protease treatments. Densely staining bands of

Figure 3.3.1 SDS PAGE gel of raw (RSB) and micronized (MIC) soyabean meal pretreated with 0 or 20 000 units of P2, P3 or P4 g^{-1} N

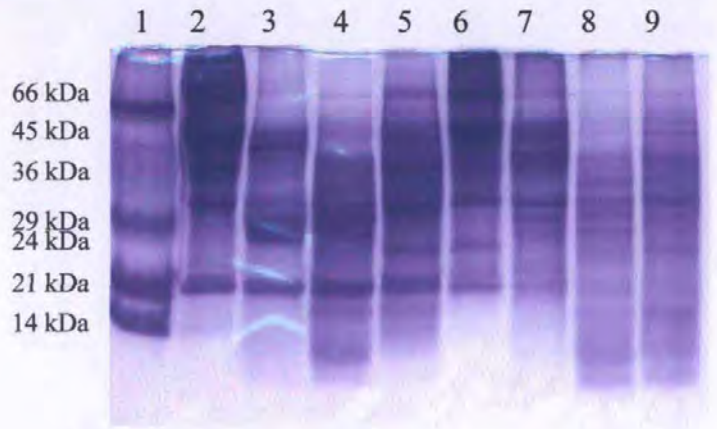


Lane 1. MW standard, 2. RSB 0 protease, 3 RSB + P2, 4. RSB + P3, 5. RSB + P4, 6. MIC 0 protease, 7. MIC + P2, 8. MIC + P3, 9. MIC + P4

Table 3.3.2. Peak area (absorbance units x mm) of main peaks from densitometry traces of Figure 3.3.1.

Soyabean meal	Raw				Micronized			
Lane	2	3	4	5	6	7	8	9
Protease treatment	0	P2	P3	P4	0	P2	P3	P4
MW band (kDa)								
80+	0.81							
75 - 79	1.69	0.54		0.19	1.29	0.46	0.26	0.36
65 - 70		0.53						
58 - 63	1.70		0.34	0.97	1.22		0.17	0.34
50 - 55								
44 - 49	3.05	1.44	0.95	2.35	1.80	0.84	0.38	0.94
36 - 43	1.11		1.38	1.75	0.39	1.16	0.46	0.66
29 - 35		1.24	0.85	0.92		0.94	0.42	0.25
21 - 27	0.88 0.10	2.09	0.61	0.13	0.07	0.13	0.14	
18 - 20	0.37	0.32	0.28	0.48	0.25			0.15
< 18								

Figure 3.3.2. SDS PAGE gel of toasted (TSD) and steam pressure cooked (SPC) soyabean meal pretreated with 0 or 20 000 units of P2, P3 or P4 g^{-1} N

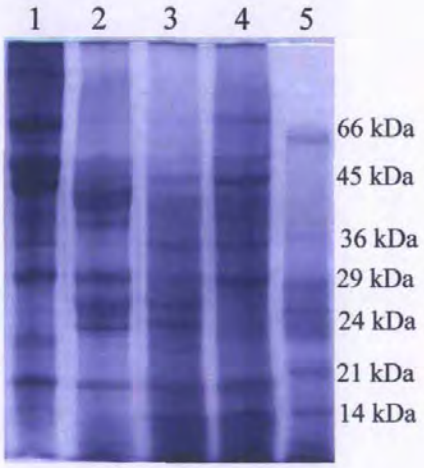


Lanes: 1. MW standard, 2. TSD 0 protease, 3. TSD + P2, 4. TSD + P3, 5. TSD + P4, 6. SPC 0 protease, 7. SPC + P2, 8. SPC + P3, 9. SPC + P4

Table 3.3.3. Peak area (absorbance units x mm) of the main peaks from densitometry traces of Figure 3.3.2)

Soyabean meal	Toasted				Steam pressure cooked			
Lane	2	3	4	5	6	7	8	9
Protease treatment	0	P2	P3	P4	0	P2	P3	P4
MW band (kDa)								
80+	1.06							
75 -79					1.64	0.56	0.06	0.08
65 - 70	2.37			0.20				
58 - 63								
50 - 55			0.15		1.41	0.87	0.07	0.30
44 - 49	3.20	1.40	0.65	1.22	2.47	1.22		0.67
36 - 43	1.82		0.60	1.92	1.27	1.05	0.53	0.95
29 - 35	1.77	1.52	2.37	1.70	1.01		1.06	
21 - 27				0.53	0.42		0.17	0.17
18 - 20	0.76	0.46	1.07	0.71	0.49	0.22	0.15	0.26
< 18			0.49				0.33	

Figure 3.3.3. SDS PAGE gel of autoclaved (AUT) soyabean meal pretreated with 0 or 20 000 units g^{-1} N of P2, P3 or P4.



Lanes 1. AUT control, 2. AUT + P2, 3. AUT +P3, 4. AUT + P4, 5. MW standard.

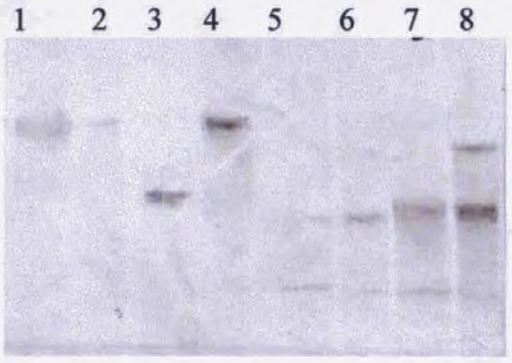
Table 3.3.4. Peak area (absorbance units x mm) of the main peaks from densitometry traces of Figure 3.3.3.

Soyabean meal	Autoclaved			
Lane	1	2	3	4
Protease treatment	0	P2	P3	P4
MW band (kDa)				
80+	0.23 0.62			
75 - 79	0.19	0.31		
65 - 70	0.89	1.72		0.30
58 - 63				
50 - 55	0.84			
44 - 49	1.04	0.53	0.54	0.57
36 - 43	0.70		0.64	0.87
29 - 35	0.48	0.52	0.51	0.52
21 - 27	0.12	0.52	0.57	1.79
18 - 20	0.38	0.74	0.93	0.53
< 16			1.05	0.96

protein of MW *circa* 45, 40 and 20 kDa were present in all of the SBM's. From their apparent MW's these were identified as the β subunit of β-conglycinin, the acidic subunit of glycinin and the basic subunit of glycinin plus KTI respectively. The β subunit of β-conglycinin and the acidic subunit of glycinin appear to be particularly resistant to hydrolysis. The β subunit (*circa* 45 kDa band) persisted in most of the protease treated SBM's with the exception of TSD and SPC treated with P3. The acidic subunit of glycinin (*circa* 40 kDa) was also present in most of the protease treated SBM's with the exception of RSB and AUT treated with P2. The staining density of the band containing the basic subunit of glycinin and KTI (*circa* 20 kDa) was reduced in MIC and SPC by all protease treatments.

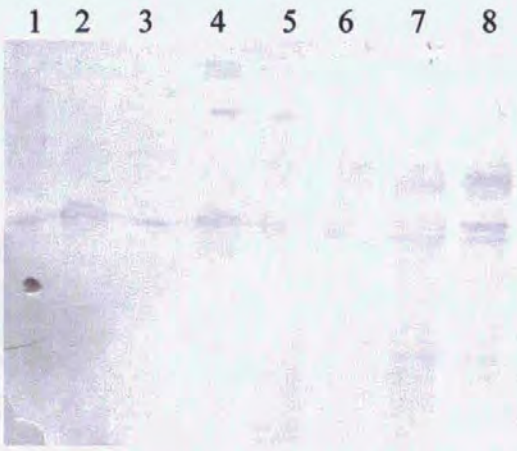
Limited success was obtained with the immunoblots. No bound antibody was detected with any of the monoclonal antibodies used. The anti-11S polyclonal antibody bound to RSB, MIC, SPC and AUT soyabean meals but none was detected in TSD soyabean meal. The Western blots for these samples are presented in Figure 3.3.4. The antibody clearly bound to two protein bands in the RSB control (Figure 3.3.4.a. lane 8). From their positions on the blot they were tentatively identified as the 60 kDa glycinin dimer and the 40 kDa acidic subunit. The lanes containing protease treated RSB show the appearance of bound antibody at sites corresponding to a lower molecular weight peptide (Figure 3.3.4.a lanes 5 - 7), indicating some hydrolysis of glycinin had taken place whilst some of the epitopes for this antibody remained intact. In the MIC control (Figure 3.3.4.a. lane 4) the anti-11S bound at one site of relatively high molecular weight (> 70 kDa), which could indicate complex formation between proteins. Treatment of MIC with P2 (Figure 3.3.4.a. lane 3) produced an epitopic peptide with lower molecular weight, whereas when treated with P3 (lane 2) and P4 (lane1) the original band persisted to a degree, which indicated at least some of the original protein remained intact in these samples. The antibody binding

Figure 3.3.4.a) Western blot showing binding of anti- 11S polyclonal antibody to raw (RSB) and micronized (MIC) soyabean meal pretreated with 0 (control) or 20 000 units of P2, P3, or P4 g^{-1} N.



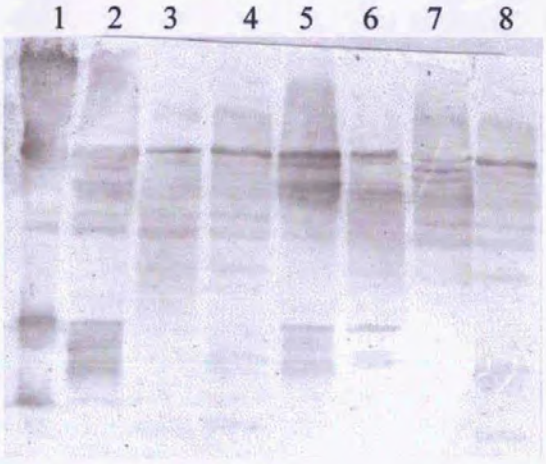
Lanes: 1. MIC+ P4, 2. MIC + P3, 3. MIC + P2, 4. MIC control, 5. RSB + P4, 6. RSB + P3, 7. RSB + P2, 8. RSB control.

Figure 3.3.4.b) Western blot showing binding of anti- 11S polyclonal antibody to steam pressure cooked (SPC) and autoclaved (AUT) soyabean meal pretreated with 0 (control) or 20 000 units of P2, P3, or P4 g^{-1} N.



Lanes: 1. AUT + P4, 2. AUT + P3, 3. AUT + P2, 4. AUT control, 5. SPC + P4, 6. SPC + P3, 7. SPC + P2, 8. SPC control

Figure 3.3.5. Western blot showing binding of anti- soy protein antibody to raw (RSB) and micronized (MIC) soyabean meal pretreated with 0 (control) or 20 000 units of P2, P3, or P4 g^{-1} N.



Lanes: 1. RSB control, 2. RSB + P2, 3. RSB + P3, 4. RSB + P4, 5. MIC control, 6. MIC + P2, 7. MIC + P3, 8. MIC + P4.

pattern for SPC (Figure 3.3.4.b. lane 8) was similar to that of RSB, with the control samples showing two discrete binding sites. However, in this case the sites were still present after treatment with P2 (Figure 3.3.4.b. lane 7), but not after treatment with P3 (lane 6) or P4 (lane 5). With AUT (Figure 3.3.4.b. lane 4) the antibody bound at high molecular weight sites (> 70 kDa), again indicating complex formation. Treatment with P2 (lane 3), P3 (lane 2) or P4 (lane 1) all resulted in the antibody binding at sites of lower molecular weight indicating partial hydrolysis of the high molecular weight complex. The anti-soy protein antibody was not specific to any particular soyabean protein and bound to a range of protein bands. An example of one of the blots using this antibody is presented in Figure 3.3.5. It was concluded that no additional information from that obtained from the gels themselves could be obtained using this antibody.

3.3.4. *Discussion and conclusions.*

On the whole, α-AN measured in the raw soya samples, was slightly lower for the control sample and for the sample treated with P2 and higher for the samples treated with P3 and P4 compared with the values obtained for enzyme inclusion levels of 20 000 units protease g^{-1} N in study 2.6. However, a different batch of raw soyabean was used in this study and the differences observed may reflect the natural variation in protein composition between different cultivars of soyabean or between conditions prevailing at the time of cultivation. In respect of the relative differences between protease treatments in the two studies, P3 consistently gave the greatest increases in α-AN in both studies, whereas P2 treatment increased α-AN production over P4 treatment in study 2.6 and the converse was true for this study. Again, this may reflect differences in protein composition between the batches of soyabean, as different cultivars are known to express different proportions of gylcinin and β-conglycinin (Nielsen 1983). However, these observations also need to be interpreted with caution, because, as Sarath *et al.* (1989) point out, the measurement of

hydrolysis products of endopeptidase activity on native protein substrates may give inconsistent results due to substrates becoming poorly defined as hydrolysis progresses.

The differences in α-AN production with the various SBM / protease combinations were probably due to conformational changes occurring in the protein during heat denaturation. Also, soyabean proteins undergo heat-induced interactions under certain processing conditions (Damodaran and Kinsella 1982; German *et al.* 1982; Utsumi *et al.* 1984; Kinsella *et al.* 1985). Conformational changes and interactions between proteins may have altered the availability of binding sites for proteases and led to the formation of different hydrolysis products compared with those obtained from unprocessed soya proteins. This was supported by the data obtained from the SDS PAGE gels. The densitometry traces showed that, for the control samples of the SBM's, there were differences in both the number of peaks and in the apparent molecular weight and staining density of the main protein bands (Tables 3.3.2 - 3.3.4). This led to differences in the apparent molecular weights of the hydrolysis products and in the number of hydrolysis products detectable by SDS PAGE. All protease treatments reduced the occurrence and / or density of the higher molecular weight peaks compared to the appropriate controls. However, some of the soya proteins, notably the β subunit of β-conglycinin and the acidic subunit of glycinin, appeared to be remarkably resistant to hydrolysis by these proteases. Although the *circa* 20 kDa band was present in many of the samples and was tentatively identified as the basic subunit of glycinin and / or KTI this may not be the case. The presence of this band did not necessarily indicate the presence of a protease-resistant peptide because it could consist of hydrolysis products of larger protein molecules.

The 'picture' of protein hydrolysis obtained from the SDS PAGE gels did not entirely support the data obtained from the α-AN assay. The gels appeared to indicate that a

greater degree of hydrolysis was obtained with MIC, SPC and AUT compared to RSB, whereas the α-AN assay indicated that the greatest degree of hydrolysis was obtained with RSB. The most anomalous results appeared to be with SPC. The gel showed treatment with P3 or P4 resulted in a greater reduction in staining density of high MW proteins and a greater increase in low MW proteins than treatment with P2, whereas, the α-AN assay showed significantly more α-AN production after treatment with P2 than with P3 or P4. A number of contibutory factors could have accounted for this and the other discrepancies between α-AN production and hydrolysis profiles on SDS PAGE gels. Firstly, heat denaturation during processing reduces protein solubility and this could have reduced the amount of α-AN extracted from the processed SBM's, especially if the hydrolysis products themselves were insoluble. This questioned the validity of drawing direct comparisons between the SBM's in respect of this assay. Although the α-AN of unsteeped SBM's was used as a covariate factor in the statistical analysis to compensate for this, it would not have compensated for the effect of insoluble hydrolysis products. Secondly, SDS PAGE has a lower detection limit of approximately 10 kDa, therefore any hydrolysis products of MW lower than 10 kDa would not have been detected. Thirdly, Coomassie blue, the dye used to stain the gels, complexes with protein by binding to basic amino acids; therefore, the amount of dye that binds to, and hence the absorbance, depended on the concentration of basic amino acids in the peptide fractions. This also means that the information obtained from the gels and densitometry traces must be interpreted with caution as the peak areas are not truly quantitative and can only give an approximate indication of relative protein concentration in each band. Despite these reservations the technique was useful in that it enabled a visual representation of the effects of the proteases on differently processed soyabean meals to be obtained.

The absence of results for the immunoblots probed with anti- soyabean lectin and anti-KTI was disappointing. This was due to the failure of the monoclonal antibodies to bind to the transferred protein. Detection of proteins by immunoblotting relies on a certain degree of renaturation of SDS denatured protein during the transfer process (Hames 1990). However, monoclonal antibodies are specific for a single epitope and, if that epitope is not sufficiently renatured during transfer, antibody binding cannot occur. The anti-11S polyclonal antibody proved more successful and this could be because polyclonal antibodies bind to more than one epitope and the chances of at least some of them being renatured during transfer is greater.

The rabbit anti-soya protein antibody obtained from Sigma showed non-specific binding of numerous protein bands. This antibody was developed in rabbits using purified soyabean protein as the immunogen and contained antibodies sensitive to a range of epitopes from all soya proteins (Sigma technical services, personal communication). Therefore, as Figure 3.3.5 shows, binding was too non-specific to interpret the effect of protease pretreatment on individual soyabean proteins.

The data presented in this study demonstrated that all three proteases were capable of hydrolysing soya protein in raw and processed soyabean meals. The differences in SDS PAGE hydrolysis profiles, observed between the different SBM/protease combinations, probably reflect changes incurred during the different processing treatments rather than the source of the soyabeans. Processing treatments affect the properties of the proteins whereas the source may affect the relative proportion of glycinin and β-conglycinin. The latter would probably manifest as differences in staining density rather than patterns of hydrolysis. The information obtained from α-AN production and SDS PAGE profiles were not in complete agreement and therefore it was not possible to determine synergistic

combinations of SBM and protease that led to the greatest degree of hydrolysis. There appeared to be no uniformity in the pattern of hydrolysis of each protease with different SBM's. In terms of protein utilization by the pig the degree of hydrolysis due to pretreatment with exogenous proteases may not be as important as the properties of the hydrolysis products and the interactions that occur between them and the pig's digestive enzymes. For example, if hydrolysis products aggregate at the low pH prevailing in the stomach this may render them less digestible than the intact protein.

3.4 The effect of enzyme pretreatment on the *in vitro* digestibility of nitrogen in raw soyabean.

3.4.1 *Introduction.*

The study reported in section 3.3 showed that different SBM / protease combinations yielded a variety of hydrolysis products. In this study an *in vitro* digestibility method was used to screen the various SBM / protease combinations for possible synergistic interactions with digestive enzymes which result in increased *in vitro* digestibility and for possible antagonistic interactions that result in decreased digestibility.

A number of methods have been published that aim to predict the *in vivo* digestibility of feedstuffs and feed ingredients for monogastric animals. Boisen and Eggum (1991) reviewed the various methods that have been developed. They concluded that the method chosen depended on the objectives of the study and in general filtration methods were of some value for predicting *in vivo* digestibility whereas dialysis techniques were better for studying the kinetics of digestion. Several filtration methods for *in vitro* digestibility have been published. Lowgren, Graham and Aman (1988) and Graham, Lowgren and Aman (1989) used a three-step method in which feed samples were digested with incubation fluids prepared from inocula of duodenal and ileal digesta and faeces of pigs. Residues were analysed for ash, nitrogen, fat and starch, after digestion and compared with undigested feed. They concluded that ileal digestibilities of starch and crude protein could be predicted from short incubation periods with duodenal digesta, but more work was needed. Decuypere, Knockaert, and Henderickx (1981) compared *in vitro* protein digestion of pig diets containing soluble and insoluble soya protein isolates using separate incubations with pepsin or trypsin. They demonstrated a correlation between *in vitro* proteolysis and *in vivo* gastric proteolysis as determined by non-protein nitrogen : total

nitrogen ratios. Cone and van der Poel (1993) and Boisen and Fernandez (1995) used a two step *in vitro* digestion method involving sequential incubation of feed samples with pepsin and pancreatin. Cone and van der Poel (1993) incubated (40° C) feed samples with pepsin (4 mg ml^{-1} 0.1M HCl) for 1.5 h followed by incubation (40° C) with pancreatin (4mg ml^{-1} pH 6.8 buffer) for 1 h. After digestion and filtration, nitrogen in the residue was determined by the Kjeldahl method and compared with N in the undigested feed. The method developed by Boisen and Fernandez (1995) used lower enzyme inclusion levels, 0.27 mg ml^{-1} reaction mixture (pH 2) and 1mg ml^{-1} reaction mixture (pH 6.8), and longer incubation times, 6 h and 18 h for pepsin and pancreatin respectively. Prior to nitrogen determination by the Kjeldahl method undigested soluble protein was precipitated with 20 % sulphosalicylic acid (SSA) and filtered. Using data from these studies and *in vivo* studies the authors generated a model that could be used to predict *in vivo* protein digestibility in feed mixtures. In a later study to predict the digestibility of energy by *in vitro* analyses Boisen and Fernandez (1997) used a three step enzymatic incubation. In this study the first two stages were essentially the same as in their previous study except that pepsin and pancreatin inclusion levels were increased to 0.69 mg ml^{-1} and 2 mg ml^{-1} reaction mixture and incubation times were reduced to 2 h and 4 h respectively. The third stage involved incubation with a multi-enzyme mix having a range of carbohydrase activities. Although the aforementioned authors have developed these methods to measure *in vitro* protein digestibility, they were all, in fact, measuring nitrogen as the end point, the underlying assumption being that nitrogen and protein are synonymous in feed ingredients.

The objective of this study was to assess the effect of different protease pretreatments of raw soyabean on the *in vitro* digestibility of protein rather than attempt to predict *in vivo* digestibility of protein as such. The assumption implicit in this objective was that, if protease pretreatment did not improve *in vitro* digestibility, it was unlikely to improve *in*

vivo digestibility. For this study the first two stages of the method developed by Boisen and Fernandez (1997) were used to assess the *in vitro* digestibility of protein in protease pretreated raw soyabean, because the shorter incubation time allowed the procedure to be completed in a day.

In the study of Boisen and Fernandez (1997) the authors found that sample size affected the variability of the results and recommended a sample size of 0.5g. They also noticed that particle size affected the variability of results with larger particle (>1 mm) sizes giving less consistent results than smaller particle sizes (<1 mm), and that this effect was most pronounced for peas, the only raw legume studied. As they are both legumes it was considered likely that raw soyabeans would be similar to peas in texture and possibly in permeability to digestive enzymes. It seemed likely that particle size would affect the *in vitro* N digestibility of raw soyabean, especially as particle size had been shown to affect the extraction of α-AN from raw soyabean. Therefore, prior to assessing the effect of protease pretreatment on the *in vitro* N digestibility (IVDN) of raw soyabean the effect of particle size on IVDN was assessed.

Previous studies, described in chapter 2, indicated that, to achieve a maximum degree of hydrolysis of raw soya protein, the optimum enzyme inclusion level was 20 000 units g^{-1} N. However, it was considered possible that lower enzyme inclusion levels, and hence a lesser degree of pre-hydrolysis of soya protein, may be required to achieve equivalent increases in IVDN. Therefore, the second objective of this study was to determine the protease inclusion levels needed to achieve maximum increases in IVDN.

3.4.2 *Materials and methods.*

Study 1

Raw soyabeans were ground with a hammer mill through a 1.5 mm screen. The ground beans were sieved through a set of graded sieves (Endicott) and separated into four fractions, 1 mm, 0.5 mm, 0.25 mm and 0.125 mm, denoted by the size of the retaining sieve. Nine replicate samples of approximately 0.5 g (weighed to an accuracy of ± 0.001 g) of each particle size were digested with pepsin and then pancreatin according to the first two stages of the method of Boisen and Fernandez (1997) (Appendix 3). Throughout each digestion period, samples were shaken continuously in a shaking water bath maintained at 39° C. After digestion, soluble protein was precipitated by adding 5 ml of 20 % sulphosalicylic acid and incubating for 30 min. at room temperature. The residues and precipitated proteins were filtered through pre-weighed filter papers (Whatman 451) and washed twice with ethanol and twice with acetone to remove fat and oil. Filter papers containing the washed residues were dried overnight in a forced air oven at 50° C and the dry weight of the residue determined. The dried residues and samples of undigested soyabean were analysed for total nitrogen using a CNS2000 analyser (Leco. UK. Ltd.) Total nitrogen contributed by pepsin and pancreatin was determined in the same way from blank digests to which no soya sample had been added. The percentage IVDN was calculated by the following equation:

$$\% \text{ IVDN} = N_s - (N_d - N_b) / N_s \times 100 \qquad \text{equation 2}$$

where N_s = nitrogen in the undigested sample; N_d = nitrogen in the digest residue; N_b = nitrogen contributed by pepsin and pancreatin in the digest mixture.

Study 2

The second study was conducted according to a two factorial design, in which factor 1 was the protease pretreatment, P2, P3 or P4 and factor 2 was the inclusion level, 0, 10 000,

20 000 or 50 000 units of protease $g^{-1}N$. Ground raw soyabean slurries were prepared according to the method in section 2.4.3. i.e. ground raw soya passed through a 0.5 mm sieve was mixed with water in a ratio of 3:1 (water:soyabean). Triplicate samples of the slurries were treated with 0, 10 000, 20 000 or 50 000 units of P2, P3 or P4 $g\ N^{-1}$. As the optimum pH of P2 was pH 4 all slurries treated with P2 were adjusted to pH 4 with 4M HCl. After steeping for 24 h three replicate samples of 1 - 2 g of each slurry were accurately weighed (± 0.001 g) into conical flasks. IVDN was determined according to the method described in the previous paragraph. Samples of the slurries prior to digestion were also analysed for total N and the dry matter content of each slurry was determined by drying to constant weight in a forced air oven at 90° C. IVDN was calculated using equation 2.

As the data generated from these studies were expressed as percentages, the distribution was checked for normality prior to analysis of variance. The data were normally distributed and therefore no transformation was undertaken. The results were analysed statistically using Minitab (Minitab inc. release 10.2, State College, Pennsylvania, 1994). Treatment means were compared using Newman - Keuls multiple range test.

3.4.3 *Results.*

Study 1

The results for IVDN of the different particle fractions are presented in Table 3.4.1. These results show that IVDN was significantly reduced ($P < 0.001$) in the 1 mm and 0.5 mm particle fractions compared to each other and to the 0.25 mm and 0.125 mm particle fractions. There was no significant difference ($P > 0.05$) in IVDN between the 0.25 mm and 0.125 mm particle sizes. This supported the findings of Boisen and Fernandez (1997) that particle size can affect the outcome of IVDN determination in some feed components.

In view of earlier findings, that particle size affected the extractability of α-AN, these results are not suprising. The data presented here indicated that the protein remained within intact cells in larger particle sizes and was inaccessible to digestive enzymes. The results of this study dictated the particle size used in the second study.

Table 3.4.1 *In vitro* nitrogen digestibility (%) of different particle sizes of ground raw soyabean.

Particle size mm				
1	0.5	0.25	0.125	s.e.d.
19.6	43.4	68.6*	64.9*	2.30

* means are not significantly different ($P > 0.05$)

Study 2

The results for IVDN of raw soyabean pretreated with increasing inclusion levels of P2, P3 or P4 are presented in Table 3.4.2. All slurries pretreated with proteases showed a significant increase in IVDN compared with the control samples. There was a significant ($P < 0.001$) increase in IVDN of 4.4 percentage units with protease inclusion levels of 10 000 units g^{-1} N compared to 0 units and of 4.6 percentage units (s.e.d. 0.44) with inclusion at 20 000 units compared with 10 000 units, but there was no further increase in IVDN when protease inclusion levels were increased to 50 000 units. P3 resulted in the best overall IVDN of 84.5 % compared with 83.3 % and 82.6 % (s.e.m.0.5) for P2 and P4 respectively. The IVDN for the control samples in this study was approximately 10 % higher than for the 0.25 mm and 0.125 mm particle fractions in the first study. However, in the first study the samples were not steeped for 24 h prior to digestion and this suggests that steeping alone may have positive effects on *in vitro* digestibility. This effect was possibly due to osmotic water uptake by the soyabean cells and subsequent leaching of

protein through partially disrupted cell walls during the 24 h steeping period. A greater degree of hydration of the soyabean particles may have aided accessibility of substrates to pepsin and pancreatin.

Table 3.4.2 In vitro nitrogen digestibility (%) of raw soyabean pretreated with 0 or 20 000 units g^{-1} N of proteases P2, P3 or P4

protease inclusion level (units g^{-1} N)	Protease P2	P3	P4	main effect: inclusion level	s.e.d.
0	78.5^{a}	77.8^{a}	77.2^{a}	77.8	0.44
10 000	82.2^{ab}	83.0^{a}	81.4^{b}	82.2	
20 000	85.8^{a1}	88.9^{1}	86.8^{a1}	86.8^{1}	
50 000	86.8^{ab1}	88.2^{b1}	86.1^{a1}	87.1^{1}	
main effect: protease	83.3	84.5	82.6		
s.e.d.	0.38				

^{a,b} means in the same row with the same superscript are not significantly different ($P > 0.05$)
^{1} means in the same column with the same superscript are not significantly different ($P > 0.05$)
s.e.d. for the interaction between protease and inclusion level = 0.75

In accordance with the results of study 2.6 a protease inclusion level of 20 000 units g^{-1}N appeared to give optimum results. Therefore, for future studies protease inclusion levels of 20 000 units g^{-1} N were used.

3.5 The effect of pretreatment with P2, P3 or P4 on the *in vitro* digestibility of nitrogen in four processed full fat soyabean meals.

3.5.1 *Introduction.*

The results of study 3.2 showed that α-amino nitrogen production in response to protease pretreatment varied for different soyabean meal/protease combinations. Electrophoresis of four differently processed soyabean meals revealed variations in the hydrolysis profiles in response to treatment with P2, P3 or P4. The observed variations could be due to heat induced interactions that take place during processing. As the processing conditions appear to affect protein hydrolysis by exogenous proteases it was considered that they may also affect hydrolysis and hence digestibility by the endogenous proteases of the pigs digestive tract. Therefore, the objective of this study was to determine the effect of protease pretreatment on the *in vitro* digestibility of nitrogen of differently processed soyabean meals.

3.5.2 *Materials and methods.*

The study was conducted using a two factor factorial experimental design. The first factor was the type of soyabean meal, RSB, SPC, MIC, TSD or AUT and the second factor was the presence or absence of 20 000 units of P2, P3 or P4 g^{-1} N. Raw soyabean and the four processed soyabean meals, SPC, MIC, TSD and AUT used in the study described in section 3.3. were sieved through a 0.5 mm sieve and slurries (3 water : 1 SBM) were prepared from the sieved fractions. Three replicate samples of each slurry were treated with 0 or 20 000 units of P2, P3 or P4 g^{-1} N and steeped for 24 h at 20° C. The slurries pretreated with P2 were adjusted to pH 4 with 4M HCl. IVDN was determined according to the method described in section 3.4.3 (study 2).

All data were checked for normality prior to analysis of variance using Minitab (Minitab inc. release 10.2, State College, Pennsylvania, 1994). Treatment means were compared using Newman - Keuls multiple range test.

3.5.3 *Results.*

The results for IVDN for raw soyabean meal and the four soyabean meals are presented in Table 3.5.1. There weresignificant differences in IVDN between some of the soyabean meals. SPC showed the greatest IVDN of 84.3 %. IVDN of RSB was significantly lower ($P < 0.05$) at 82. 4% (s.e.m. 0.5). MIC and AUT both gave significantly lower ($P < 0.01$) values for IVDN than SPC or RSB (78.4 % and 77.9 % s.e.m. 0.5 respectively), but there was no significant difference between them. TSD gave a significantly lower ($P < 0.001$) value of 72.7 % IVDN than all of the others. In respect of protease treatment there were no overall significant differences between P2, P3 and P4 but they significantly increased ($P < 0.001$) IVDN by 6.5%, 7.4 % and 7.2 % respectively compared to the controls. Analysis of the interaction between protease treatment and soyabean meal showed the interaction to be highly significant ($P < 0.001$) and although all protease treatments significantly increased IVDN in all soyabean meals compared to the controls no single protease gave consistently better results with all SBM's. This was similar to the results obtained for α-AN production reported in section 3.3. The results of study 3.3 and the present study were analysed to determine if there was any correlation between α-AN production and IVDN. The correlation coefficients for IVDN and α-AN with protease treatment were 0.42, - 0.89, 0.93, 0.72 and 0.46 for RSB, SPC, MIC, TSD and AUT respectively.

Table 3.5.1. Factorial analysis of in vitro Nitrogen digestibility (%) of raw (RSB), steam pressure cooked (SPC), micronized (MIC), toasted (TSD) and autoclaved (AUT) soyabean meals steeped for 24 h at 20° C with 0 or 20 000 units g^{-1} N of proteases P2, P3 or P4.

	Protease					
Soyabean meal	0	P2	P3	P4	Main effect: soyabean meal	s.e.d.
RSB	76.4^{1}	84.0^{ab1}	86.3^{b1}	82.9^{a1}	82.4	0.71
SPC	80.3	84.0^{a1}	84.9^{a12}	87.8	84.3	
MIC	74.7^{1}	79.6^{a2}	83.0^{2}	77.5^{a}	78.4^{1}	
TSD	67.8	73.5^{a}	74.4^{a}	74.8^{a}	72.7	
AUT	70.1	81.1^{a2}	78.1	82.3^{a1}	77.9^{1}	
Main effect: Protease	73.9	80.4^{a}	81.3^{a}	81.1^{a}		
s.e.d.	0.63					

^{1,2} means in the same column with the same superscript are not significantly different $P > 0.05$
^{a,b} means in the same row with the same superscript are not significantly different $P > 0.05$
s.e.d. for the interaction between soyabean meal and protease = 1.42

3.5.4 *Discussion.*

IVDN varied considerably between the untreated SBM controls, ranging from 67.8 % for TSD to 80.3 % (s.e.m. 1.42) for SPC. This could be due to either the processing conditions imposed, or to the source of the soyabeans prior to processing, or a combination of these factors. Heat induced interactions occur between soyabean proteins as the subunits of glycinin and β-conglycinin dissociate and form macro-complexes during heating (German *et al.* 1982). This may result in the formation of protein complexes in which the binding sites for digestive enzymes are exposed to varying degrees. Processing also affects protein solubility (Wright 1981) which, in turn, could affect the availability of scissile bonds for degradation by digestive proteases. Ikeda *et al.* (1995) demonstrated differences in *in vitro* peptic digestion of a number of oriental soyabean foods and concluded that the chemical form of soya protein imparted by processing conditions affects digestibility.

The data presented here indicate that the pigs' endogenous digestive enzymes are equally as capable of digesting raw soyabean as they are processed soyabean meals. This implies that the poor performance observed when raw soyabean is fed to pigs is mainly due to the adverse effects of antinutritional factors rather than any inherent indigestibility of the protein itself. The levels of serine protease inhibitors in the raw beans did not appear to reduce *in vitro* digestibility compared with the processed soyabean meals. This could have been due to a number of factors. The levels of serine proteases in the pancreatic stage of digestion could have been in excess such that there was sufficient of these enzymes left uninhibited to achieve protein digestion. Native soyabean protein may have been even more digestible in the absence of any serine protease inhibitors or the serine protease inhibitors present may have been inactivated by the hydrolytic action of pepsin.

All protease pretreatments significantly increased IVDN in all SBM's compared to the controls. However, no protease gave consistently better results than any other. This reflects the results obtained for α-AN production. Although a positive correlation between IVDN and α-AN was expected this was only really apparent in MIC in which the correlation coefficient between IVDN and α-AN was 0.93. The correlation between IVDN and α-AN for SPC was negative (-0.89). The inconsistencies observed here probably reflect the differences in protein conformation between the processed soya bean meals.

Boisen and Fernandez (1995) obtained a value of 92.9 % IVDN for soyabean meal. This is considerably higher than the values obtained in this study. However, as this study shows, the method of processing does appear to effect IVDN. The type of SBM used in the study of Boisen and Fernandez (1995) was not stated. Therefore the value for IVDN they obtained may be due to the variability which appears to exist between differently processed SBM's. Having said that, the values obtained for IVDN of the control samples in the

present study are rather low. It could be expected that values for IVDN approach those for true ileal digestibilities (Boisen and Eggum 1991) and would therefore, be much higher than apparent ileal digestibilities. Marty *et al.* (1994) obtained apparent ileal N digestibilities of 71.7 % and 69.5 % for micronized and toasted full fat soyabean respectively, and Qin *et al.* (1996) obtained an apparent ileal N digestibility of 82 % for steam pressure cooked full fat soyabean. The rather low values obtained for IVDN in the present study probably reflect shortcomings in the method used. The earlier (1995) method of Boisen and Fernandez (1995) was developed specifically to estimate IVDN, whereas the latter method (Boisen and Fernandez 1997), which was adapted for this study, was developed as a three stage enzymatic digestion to measure total tract digestibility of energy. In the former method incubation times were longer which may have resulted in a higher degree of protein digestion. Also the filtration step in the method of Boisen and Fernandez (1995) involved filtering samples though glass filters with the addition of celite as a filter aid, whereas in the present study samples were filtered through filter paper. It was possible that the filter paper used retained more of the smaller peptides or non-protein nitrogen than the filtration system used by Boisen and Fernandez (1995). However, as all samples were processed identically it was felt that the adapted method used here presented a valid comparison between different SBM's and protease treatments.

The data presented here showed that *in vitro* digestibility of nitrogen was improved by pretreatment with exogenous proteases. Although the results cannot be extrapolated to an *in vivo* situation because there is no way of accounting for endogenous nitrogen loss, it provides a useful method for screening exogenous enzymes and the polypeptide products of partial hydrolysis against digestive enzymes.

3.6. The effect of protease pretreatment of soyabean meals on the *in vitro* digestibility of nitrogen and protein solubility at different pH's.

3.6.1 *Introduction*

The previous study demonstrated that *in vitro* nitrogen digestibility can be increased by pretreatment of soyabean meals with proteases in an aqueous medium. The differences observed between IVDN of the different SBM's and the varied responses to protease pretreatment could be a function of heat induced conformational changes in soyabean protein. One of the ways heat induced changes in protein manifests is in a reduction in protein solubility. This property forms the basis of the protein dispersibility and nitrogen solubility indices used in the soyabean processing industry as a determinant of product quality (Waggle and Kolar 1978; Wright 1981). Both of these methods determine protein solubility in water at neutral pH. However, in terms of digestibility, the solubility of protein at the pH prevailing in the stomach may be of more importance. It is well documented that the pH prevailing in the stomach of the weaner pig is relatively high, *circa* pH 4 (Makkink, Bernsten, op den Kamp, Kemp and Verstegen 1994; Aumaitre, Peiniau and Madec 1995; Roth and Kirchgessner 1998) compared to that of the older pigs where gastric pH is generally about pH 2 (Kidder and Manners 1978). This means the weaner pig is disadvantaged in respect of protein digestion on two counts. Firstly, the gastric pH is out of the optimum range for the activity of porcine pepsin, which has two pH optima pH 2 and pH 3.5 (Kidder and Manners 1978). Secondly, the solubility of soyabean protein is at its lowest at pH 4 and this may impair digestion of soya protein by limiting the access of pepsin to its substrate. Carbonaro *et al.* (1997) suggest that protein digestibility of a number of legume proteins may be adversely affected by the formation of insoluble aggregates during cooking. This suggestion is supported by a study undertaken by Decuypere, Knockaert and Henerickx (1981) in which the *in vitro* and *in vivo* digestibility

of soluble and insoluble soya protein isolates was compared. They found that both *in vitro* digestibility, determined by peptic and subsequent tryptic digestion, and *in vivo* hydrolysation of protein in the stomachs of gastric fistulated 5 week old pigs was significantly increased in pigs fed diets containing soluble soya protein compared with insoluble soya protein.

In the previous study the partial hydrolysis of raw and processed soyabean protein increased IVDN under conditions simulating a gastric pH of pH 2. The objectives of the present study were:-

- to determine whether protease pretreatment had similar effects on IVDN under pH conditions prevailing in the stomach of the young pig
- to investigate the effect of protease pretreatment on the solubility of soya protein at pH 2 and pH 4
- to investigate the effect of peptic hydrolysis on trypsin inhibitor activity of raw soyabean.

3.6.2 *Materials and methods.*

Study 1

A study was conducted according to a three factor factorial design to investigate the effect of pH of peptic digestion and protease treatment on soyabean meals. The first factor was the type of soyabean meal, RSB, SPC, MIC, TSD or AUT, the second factor was the presence or absence of 20 000 units of P2, P3 or P4 g^{-1} N, the third factor was the pH of peptic digestion, pH 2 or pH 4. Ground soyabean meal slurries were prepared and pretreated with P2, P3 or P4 according to the method described in section 3.5.2. IVDN was performed on the slurries according to the method described in section 3.4.2. The procedure was repeated with the peptic digestion stage adjusted to pH 4 instead of pH 2.

Study 2

For the protein solubility assay three replicate samples of 1 g of each slurry were transferred to conical flasks, 50 ml of the phosphate buffer used in the peptic digest added, and the pH adjusted to pH 2 with 4M HCl. The flasks were shaken continuously for three hours on a rotating platform mixer (Rotamix Sorval instruments). This procedure was repeated with the pH of the buffer adjusted to pH 4 instead of pH 2. Triplicate samples of each soluble protein extract were centrifuged at 5000 r.p.m and the supernatants used to determine the amount of soluble protein using a protein assay kit (Biorad). Three replicate aliquots of 400 µl of an appropriate dilution of each sample were pipetted into wells of microtitre plates and 50 µl of Bradfords reagent added. After 20 min incubation at ambient temperature the absorbance at 595 nm was read on a microtitre plate reader (ELX 800 Bio-Tek Instruments Inc. Vermont USA). A calibration standard (bovine serum albumin) was included in each plate according to the instructions provided with the assay kit.

Study 3.

A two factor factorial experimental design was used to assess the effect of pepsin digestion on trypsin inhibitor activity of raw soyabean. The first factor was protease pretreatment, 0, P2, P3 or P4 and the second factor was the pH used in the peptic digestion, pH 2 or pH 4. Raw soyabean slurries were prepared and pretreated with no protease or 20 000 units of P2, P3 or P4 g^{-1} N as in previous studies. After incubation for 24 h at 20° C three replicate samples of approximately 0.5 g were accurately weighed (± 0.001 g) into conical flasks and subjected to pepsin digestion at pH 2 or pH 4 according to the pepsin digestion stage described in section 3.3. Further samples of each slurry were taken for dry matter determination by drying in a forced air oven at 90° C to constant weight. After digestion the pH of each sample was adjusted to pH 8.2 with M NaOH, to halt digestion, and shaken

for 2 h. The samples were centrifuged and the supernatants assayed for trypsin inhibitor activity according to the method of Smith *et al.* (1980).

All data were checked for normality prior to statistical analysis. The data obtained from studies 1 and 2 were analysed by analysis of variance and regression analysis. The data obtained from study 3 was analysed by analysis of variance. All analyses were conducted using Minitab (Minitab inc. release 10.2, State College, Pennsylvania, 1994) and treatment means were compared using Newman - Keuls multiple range test.

3.6.3 *Results.*

The results of the factorial analyses of the effect of peptic digestion at pH 2 or pH 4 and protease pretreatment on IVDN are presented in Table 3.6.1. IVDN was reduced by 22.4 (s.e.d. 0.37) percentage points ($P < 0.001$) as a result of conducting peptic digestion at pH 4 rather than pH 2 for all soyabean meals. Protease pretreatment significantly increased ($P < 0.001$) IVDN for all soyabean meals at both pH's. The regression equation for effects of pH of peptic digestion, protease pretreatment and type of soyabean meal on IVDN was

$$IVDN = 97.3 - 11.2\ pH + 2.31\ protease - 0.450\ SBM \qquad \text{Equation 3}$$

($r^2 = 0.74$, RSD = 6.9)

Analysis of S^2 values for the regression equation showed that pH accounted for 92% of the explained variation and protease pretreatment and type of soyabean meal contributed 8 % and 0.3 % respectively.

Table 3.6.1 Factorial analyses of *in vitro* Nitrogen digestibility (%) at two gastric pH's of raw (RSB), steam pressure cooked (SPC), micronized (MIC), toasted (TSD) and autoclaved (AUT) soyabean meals steeped for 24 h at 20° C and pretreated with 0 or 20 000 units g^{-1} N of protease P2, P3, P4.

a) interaction between type of soyabean meal and pH

	Soyabean meal						
	RSB	SPC	MIC	TSD	AUT	Main effect: pH	s.e.d.
pH 2	82.4	84.3	78.5[a]	72.7	77.9[a]	79.1	0.37
pH 4	49.9	58.3[a]	58.4[a]	64.2	52.7	56.7	
Main effect: soya	66.1[b]	71.3	68.5[a]	68.5[a]	65.3[b]		
s.e.d.	0.58						

s.e.d. for the interaction between SBM and pH = 0.82

b) interaction between protease pretreatment and type of soyabean meal

	Protease					
SBM	0	P2	P3	P4	Main effect: soya	s.e.d.
RSB	59.7	70.[1a1]	69.5[a2]	65.2	66.1[2]	0.58
SPC	64.1[1]	73.3[a]	73.1[a1]	74.5[a]	71.3	
MIC	62.6[1]	70.6[a1]	71.4[a12]	69.2[a1]	68.5[1]	
TSD	62.3[1]	69.5[a1]	69.4[a2]	72.7	68.5[1]	
AUT	57.0.	68.6[a1]	65.9	69.7[a1]	65.3[2]	
Main effect: protease	61.13	70.43[a]	69.85[a]	70.26[a]		
s.e.d.	0.52					

s.e.d. for the interaction between SBM and protease = 1.17

c) interaction between protease pretreatment and pH

	Protease					
	0	P2	P3	P4	Main effect: pH	s.e.d.
pH 2	73.9	80.4[a]	81.3[a]	81.1[a]	79.1	0.37
pH 4	48.4	60.5[a]	58.4[b]	59.5[ab]	56.7	
Main effect: protease	61.1	70.4[a]	69.9[a]	70.3[a]		
s.e.d.	0.52					

s.e.d. for the interaction between protease and pH = 0.74

[a,b] means in the same row with the same superscript do not differ significantly $P > 0.05$

[1,2] means in the same column with the same superscipt do not differ significantly $P > 0.05$

Study 2

The factorial analyses for the effect of pH, protease pretreatment and type of soyabean meal on protein solubility are presented in Table 3.6.2. Protein solubility was significantly decreased ($P < 0.001$) from 64.5 at pH 2 to 6.9 (s.e.m. 0.36) mg g^{-1} SBM at pH 4 overall. The protein solubility of the processed soyabean meals ranged from 26.4 to 31.7 mg g^{-1} SBM and was significantly lower ($P < 0.001$) than that of raw soyabean, 60.6 mg g^{-1} SBM.. Pretreatment with P2 significantly decreased ($P < 0.001$) protein solubility by 27.2 (s.e.d. 0.71) mg g^{-1} SBM to compared to the untreated controls, whereas pretreatment with P3 or P4 significantly increased ($P < 0.001$) protein solubility by 13.1 and 22.1 mg g^{-1} SBM respectively. The effects of protease addition were apparent for all soyabean meals at both pH's. The regression equation for the effects of pH, protease pretreatment and type of soyabean meal on protein solubility was:

Protein solubility = 128 - 28.8 pH - 6.67 SBM + 6.33 protease. Equation 4

($r^2 = 0.55$, RSD = 28.4)

Analysis of S^2 values for the regression equation showed that pH accounted for 82 % of the explained variation and type of SBM and protease pretreatment accounted for 9 % and 8.7 % respectively.

The correlation coefficient between IVDN and protein solubility was 0.66.

Table 3.6.2. Factorial analyses of protein solubility (mg soluble protein g^{-1} SBM) at pH 2 and pH 4 of raw (RSB), steam pressure cooked (SPC), micronized (MIC), toasted (TSD) and autoclaved (AUT) soyabean meals steeped for 24 h at 20° C and pretreated with 0 or 20 000 units g^{-1} N of protease P2, P3, P4.

a) interaction between type of soyabean meal and pH

	Soyabean meal						
	RSB	SPC	MIC	TSD	AUT	Main effect: pH	s.e.d
pH 2	110.3	57.2^{a}	54.5^{a}	45.3	55.3^{a}	64.5	0.5
pH 4	10.8	6.2^{ab}	5.8^{ab}	7.4^{a}	4.4^{b}	6.9	
Main effect: soya	60.6	31.7^{a}	30.2^{ab}	26.4	29.9^{b}		
s.e.d.	0.79						

s.e.d. for the interaction between SBM and pH = 1.13

b) interaction between protease pretreatment and type of soyabean meal

	Protease					
SBM	0	P2	P3	P4	Main effect: soya	s.e.d.
RSB	68.5^{a}	17.3	71.5^{a}	84.9	60.6	0.79
SPC	34.3	3.8^{1}	43.5^{a12}	45.1^{a1}	31.7^{1}	
MIC	26.3^{1}	3.7^{1}	40.9^{1}	49.7	30.2^{12}	
TSD	23.4^{1}	3.9^{1}	31.8	46.3^{1}	26.4	
AUT	16.0	3.8^{1}	46.5^{2}	53.2	29.9^{2}	
Main effect: protease	33.7	6.5	46.8	55.8		
s.e.d.	0.71					

s.e.d. for the interaction between SBM and protease = 1.59

c) interaction between protease pretreatment and pH

	Protease					
	0	P2	P3	P4	Main effect: pH	s.e.d.
pH 2	62.8	10.1	83.6	101.6	64.5	0.5
pH 4	4.6	2.3	10.1^{a}	10.1^{a}	6.9	
Main effect: protease	33.7	6.5	46.8	55.8		
s.e.d.	0.71					

s.e.d. for the interaction between protease and pH = 1.01

^{a,b} means in the same row with the same superscript do not differ significantly $P > 0.05$

^{1,2} means in the same column with the same superscript do not differ significantly $P > 0.05$

The results of the trypsin inhibitor assay on the pepsin digests are presented in Table 3.6.3. Trypsin inhibitor activity was significantly reduced ($P < 0.001$) by peptic digestion at both pH 2 and pH 4 compared with the trypsin inhibitor activity of the soyabean meal prior to steeping or protease pretreatment. Pretreatment with P3 and P4 significantly reduced trypsin inhibitor activity compared with no protease treatment or treatment with P2. The interaction between protease-treatment and the pH of peptic digestion was highly significant ($P < 0.001$). The greatest reduction in trypsin inhibitor activity was observed with raw soyabean that had been pretreated with P4 with peptic digestion at pH 2.

Table 3.6.3 Factorial analysis of Trypsin Inhibitor Activity (mg trypsin inhibited g^{-1} soyabean dry matter) after steeping raw soyabean meal for 24 h at 20° C with 0 or 20 000 units g^{-1} N of protease P2, P3 or P4 followed by digestion with pepsin at pH 2 or pH 4.

	Protease					
Pepsin digest pH	0	P2	P3	P4	Main effect: pH	s.e.d.
prior to treatment	26.7	26.7	26.7	26.7	26.7	0.44
pH 2	10.6[a]	16.8	12.1[a]	6.4	11.5	
pH 4	22.5	15.3[a]	15.7[a]	17.8	17.8	
Main effect: protease	19.9[a]	19.6[a]	18.2	17.0		
s.e.d.	0.50					

s.e.d. for the interaction between protease and pH = 0.87

[a] means with the same superscript in the same row are not significantly different ($P < 0.05$)

3.6.4. *Discussion.*

The factor having the greatest effect on IVDN was the pH of peptic digestion, with IVDN being significantly reduced by peptic digestion at pH 4 compared to pH 2 in all cases. This was not suprising as pH 4 is outside the optimum range for pepsin activity. Protease pretreatment increased IVDN at both pH 4 and pH 2 compared with the controls. With P2 this effect may have been attributable to the fact that its optimum pH was pH 4 and

therefore it was likely to remain active during the peptic digestion stage. This could have contributed to P2 having the greatest effect overall in this study compared with P3 having the greatest overall effect in the previous study. P3 and P4 have pH optima around neutral and therefore the increase in IVDN observed at pH 4 with these enzymes was unlikely to be due to any considerable retention of activity during peptic digestion. It was considered that the increases in IVDN due to protease pretreatment could be explained in part by the formation of soluble hydrolysis products. The presence of soluble low molecular weight peptides in the stomach may increase the efficiency of protein hydrolysis by pepsin due to favourable presentation of binding sites and facilitation of protein / pepsin interactions. When the results of the effect of protease treatment on protein solubility were analysed this did indeed appear to be the case for pretreatment with P3 and P4. Pretreatment with both of these enzymes increased protein solubility for all soyabean meals at pH 2 and pH 4, although solubility at pH 4 was still significantly reduced in all cases. P2 on the other hand appeared to drastically reduce protein solubility at both pH 2 and pH 4 for all SBM's. This appeared to be at variance with the results obtained for P3 and P4 and with the suggestion made by Decuypere *et al.* (1981) that soluble protein should be more digestible than insoluble protein simply because solubility facilitates enzyme/ substrate contact. However, there may be an explanation for the anomalous results observed with P2. The reagent used in the protein assay, Bradfords reagent, uses the dye Coomassie blue to detect protein. Coomassie blue binds to basic amino acids in peptide chains of greater than 8 - 10 amino acids (Biorad Technical Services, personal communication). Information supplied by Finnfeeds indicated that P2 was an exoprotease and cleaves di and tri-peptides and free amino acids from the ends of peptides whereas P3 and P4 were endopeptidases and cleave large molecules into smaller polypeptides (H. Schulze Finnfeeds International, Marlborough, personal communication). If this was the case, then the hydrolysis products of P2 would have gone undetected in the reaction mixture giving an apparent reduction in

protein solubility which was in fact due to a reduction in detectable protein. Therefore, the data for protein solubility was re-analysed omitting the data for P2 as this was deemed invalid. These data are presented in Table 3.6.4. Although the values for the main effects of SBM and pH were increased in this analysis there was no change in the significance of any effects due to omission of the data for P2. The correlation coefficient for IVDN and protein solubility with the data for P2 omitted was 0.83, which suggested there was a relationship between protein solubility and IVDN.

The results of the trypsin inhibitor assay showed that TIA was reduced by 60% after digestion with pepsin at pH 2. Pretreatment of RSB with P3, prior to peptic digestion at pH 2, did not result in any further reduction in TIA, whereas pretreatment with P4 resulted in a further 16% decrease. These results suggested that P4 acted in synergy with pepsin in reducing TIA whereas P3 did not. Pretreatment of RSB with P2 prior to peptic digestion at pH 2 increased TIA by 59% compared to peptic digestion with no prior protease treatment. Therefore, P2 would appear to be antagonistic towards the action of pepsin on KTI and/or BBI at pH 2. When peptic digestion was conducted at pH 4 all protease treatments showed a significant reduction in TIA compared to the control samples. For P3 and P4 the reduction in TIA was 41% and 33% respectively. This was similar to that achieved in study 2.7, where pretreatment of RSB with P3 and P4 alone, produced a reduction in TIA of 39% and 35% respectively. This suggests that, when peptic digestion was conducted at pH 4, the reduction in TIA was mainly due to the action of the proteases rather than pepsin. Treatment with P2 followed by peptic digestion at pH 4 reduced TIA by 42% in the present study compared with 30% in study 2.7. This was possibly due to the continued action of P2 during the peptic digestion stage at pH 4.

Table 3.6.4. Factorial analyses of protein solubility (mg soluble protein g^{-1} SBM) at pH 2 and pH 4 of raw (RSB), steam pressure cooked (SPC), micronized (MIC), toasted (TSD) and autoclaved (AUT) soyabean meals steeped for 24 h at 20° C and pretreated with 0 or 20 000 units g^{-1} N of protease P3 or P4.

a) interaction between type of soyabean meal and pH

	Soyabean meal						
	RSB	SPC	MIC	TSD	AUT	Main effect: pH	s.e.d
pH 2	137.5	74.2[a]	71.2[a]	58.9	71.5[a]	82.7	0.66
pH 4	12.4	7.7	6.8	8.9	5.6	8.3	
Main effect: soya	75.0	41.0[a]	39.0[ab]	33.9	38.5[b]		
s.e.d.	1.05						

s.e.d. for the interaction between SBM and pH = 1.49

b) interaction between protease pretreatment and type of soyabean meal

	Protease				
SBM	0	P3	P4	Main effect: soya	s.e.d.
RSB	68.5[a]	71.5[a]	84.9	75.0	1.05
SPC	34.3	43.5[a12]	45.1[a1]	41.0[1]	
MIC	26.3[1]	40.9[1]	49.7	39.0[12]	
TSD	23.4[1]	31.8	46.3[1]	33.9	
AUT	16.0	46.5[2]	53.2	38.5[2]	
Main effect: protease	33.7	46.8	55.8		
s.e.d.	0.82				

s.e.d. for the interaction between SBM and protease = 1.83

c) interaction between protease pretreatment and pH

	Protease				
	0	P3	P4	Main effect: pH	s.e.d
pH 2	62.8	83.6	101.6	82.7	0.66
pH 4	4.6	10.1[a]	10.1[a]	8.3	
Main effect: protease	33.7	46.8	55.8		
s.e.d.	0.82				

s.e.d. for the interaction between protease and pH = 1.15

[a,b] means in the same row with the same superscript do not differ significantly $P > 0.05$

[1,2] means in the same column with the same superscript do not differ significantly $P > 0.05$

3.7 *Conclusions*

The studies described in this chapter demonstrated that all three proteases were capable of hydrolysing soya protein in a range of processed soyabean meals. The interactions between differently processed meals and the proteases resulted in slightly different hydrolysis products. This was probably due to conformational changes and heat induced interactions during processing altering the availability of binding sites for the proteases. All protease pretreatments increased *in vitro* nitrogen digestibility in raw soyabean and all of the processed soya bean meals. For soyabean meals treated with P3 and P4 this was probably due to the presence of smaller, more soluble molecular weight peptides in the digesta which facilitated further hydrolysis by digestive enzymes. For soyabean meals treated with P2 the release of di and tri-peptides and free amino acids from the soya protein due to the action of P2 would in itself have contributed to *in vitro* digestibility. Whether the residual polypeptides resulting from cleavage with P2 were any more or less digestible than the untreated protein remains uncertain. Although it has been suggested that native legume proteins are inherently less digestible by mammalian digestive enzymes than heat treated legume proteins (Nielsen *et al.* 1988), it was apparent from this study that raw soyabean meal is equally as digestible by the pig's digestive enzymes as the processed soyabean meals. Having said that, steeping alone appeared to increase the digestibility of raw soyabean meal and this could be due to the activation of endogenous enzymes in the ground meal. Endogenous proteases activated by the uptake of water could increase the digestibility of soyabean protein by partial hydrolysis.

Although all the protease treatments increased protein digestibility *in vitro* the results cannot necessarily be extrapolated to an *in vivo* situation. However, the *in vitro* digestibility method provided a useful way of screening for favourable synergistic reactions between soyabean meals, protease pretreatments and digestive enzymes. The best overall

increase in *in vitro* N digestibility was observed with protease P3. However, the most favourable combination of soyabean meal / protease was steam pressure cooked soyabean meal and P2. On the other hand, P4 gave the greatest reduction in trypsin inhibitor activity after peptic digestion at pH 2.

Chapter 4.

The effect of protease pretreatment of raw and processed soyabean meal on the growth performance of pigs.

4.1. Introduction

It is well documented that grower and finisher pigs do not utilise raw soyabean well (Jiminez *et al.* 1963; Combs *et al.* 1967; Young 1967; Vandergrift *et al.* 1983; Crenshaw and Danielson 1985b). . This has been attributed to the presence of antinutritional factors in raw soyabeans that are destroyed by heat processing, notably the serine protease inhibitors (SPI). Yen *et al.* (1974) and Cook *et al.* (1988) showed that pigs fed low SPI varieties of raw soyabean performed better than those fed high SPI varieties but not as well as pigs fed processed soyabean. Laboratory studies reported in section 2.7 demonstrated that pretreatment of raw soyabean meal by steeping for 24 h at 20° C in the presence of 20 000 units g^{-1} N of P2, P3 or P4 significantly reduced trypsin inhibitor from 27.6 (untreated control) to 20.0, 17.2 and 18.4 mg trypsin inhibited g^{-1} RSB (DM) respectively. With an inclusion rate of 30 % RSB in a grower pig diet these figures equate to 8.3, 6.0, 5.2 and 5.5 mg trypsin inhibited g^{-1} diet respectively. Although these values are above the threshold level of 2.5 mg trypsin inhibited g^{-1} diet postulated by Schulze *et al.* (1993a), they do represent a reduction in total SPI content and Schulze *et al.* (1993a) showed a dose response relationship to increasing levels of SPI above the threshold level. Therefore, in reducing total SPI content of the diet there may be some benefit in growth performance of feeding protease treated RSB to growing pigs.

The laboratory studies reported in chapter 3 demonstrated that all three proteases investigated partially hydrolysed soyabean protein (3.3) and increased *in vitro* digestibility of nitrogen in raw soyabean and a number of full fat soya bean meals (3.5). However, few

studies have been undertaken on the effect of protease pretreatment of raw soyabean or processed soyabean meals on the growth performance of pigs. The studies that have been undertaken have concentrated on newly weaned pigs. Caine *et al.* (1997b) found no significant differences in apparent ileal digestibility of amino acids in pigs weaned at 20 days fed diets containing soyabean meal treated with an acid protease (*Bacillus subtilis* subtilisin) compared with untreated soyabean meal. In studies comparing soyabean meal treated with an acid protease or an alkaline protease, Hessing *et al.* (1996) and Rooke *et al.* (1998) found that the acid protease treated meal significantly increased liveweight gain in four week old weaned piglets. Both Hessing *et al.* (1996) and Rooke *et al.* (1998) concluded that protease treatment of soyabean meal could have potential benefits for the performance of weaner pigs.

Processed soyabean meals appear to be well utilised by grower and finisher pigs. Data on the apparent ileal digestibility of nitrogen in full fat soyabean meals vary between 69 % (Knabe *et al.* 1989) and 82 % (Grala *et al.* 1998a) depending on the processing treatment. NRC (1998) gives a value for true digestibility of lysine of 86 % for full fat soya. Due to the ubiquitous use of soyabean meals in animal feeds even small increases in the digestibility of protein in soyabean meals could have cost benefits.

The dietary protein requirements for pigs are usually expressed in g lysine MJ DE^{-1}, because lysine is generally the first limiting amino acid in pig diets and the maximum lean tissue deposition rate in the growing pig is dependent on the energy provided in the diet (NRC 1998). With a diet of a given energy density, protein deposition increases linearly with increasing protein concentration until the pigs' maximum rate of protein accretion is attained. Further increases in protein provision do not result in increased lean tissue deposition (Edwards and Campbell 1993) and surplus protein is deaminated. There is also

an upper limit to protein retention, independent of energy provision in the diet, which is influenced by the genotype, age and sex of the animal (Moughan 1998). In order to elicit a response from the pig due to any increase in protein digestibility resulting from pretreatment of soyabean meals with exogenous proteases, the lysine : energy ratio was reduced in the diets used in these studies. Thus diets were formulated to given energy values, and the lysine concentration reduced below requirement. Published figures for the lysine:energy requirement of growing pigs (20 - 50 kg) vary between 0.75 g MJ DE^{-1} (SCA 1987) to 0.84g MJ DE^{-1} (Close 1991) and for finishing pigs (50 - 90 kg) they vary between 0.65 g MJ DE^{-1} (SCA 1987) to 0.75 g MJ DE^{-1} (Whittemore 1993). For the purposes of these studies an intermediate value between the two was taken and diets formulated to contain 85 - 90 % of this value. Dietary lysine:energy ratios are stated in the methods section for each feeding trial. Ratios of all other amino acids in the diet formulations were adjusted in relation to lysine in accordance with the amino acid ratio of 'ideal' protein (Cole 1996).

Soya protein does not provide an ideal balance of amino acids for the pig, and is limiting with respect to lysine. The protein value (V) of feed ingredients can be estimated relative to the value of the ideal protein from the level of lysine in the ingredient (Whittemore 1993) using the equation:

$$V = \text{g lysine kg}^{-1} \text{ crude protein} / 0.07 \text{ (g lysine g}^{-1} \text{ ideal protein)} \qquad \text{equation 5}$$

For raw or processed full fat soyabean meal with a lysine content of 2.2% and a crude protein content of 34.5% this is 0.91. Although the protein value for raw and processed soyabean meal is identical the apparent ileal digestibility of lysine for raw soyabean is 57% compared with 80% for steamed soyabean (Yin *et al.*1993). In formulating the diets for the present studies a decision was made not to use synthetic amino acids to balance the protein in the diet. Any improvement in digestibility due to enzyme pretreatment should be

reflected in increased lysine availability and hence increased performance as the lysine : energy ratio would be increased. Also there is evidence that synthetic amino acids are not fully utilized in meal fed pigs (Batterham 1985), as they may be absorbed ahead of amino acids digested from dietary protein sources and catabolized rather than anabolized and it was felt that this would not be a controllable factor in this study.

Whilst enzyme treatments can do nothing to increase the biological value of protein, all three of the proteases investigated partially hydrolysed soya protein and increased *in vitro* digestibility of nitrogen in raw soyabean meal and processed full fat soyabean meals. The objective of the studies reported here was to investigate whether the effects of the proteases observed in the laboratory studies were reflected in increased utilisation of raw and processed soyabean meal in the pig.

Proteases P3 and P4 were selected for study *in vivo*. In both feed trials it was intended that enzyme pretreatment of dietary components would be undertaken on the farm and although P2 gave good results in the *in vitro* digestibility screening method its use had a number of drawbacks pertaining to its use *in situ*. The optimum pH of P2 is approximately pH4 which would have necessitated acidifying the soyabean prior to enzyme treatment, also it was in a very fine powder form which would have presented handling difficulties and increased the hazard of possible allergenic reactions. Furthermore, information from the suppliers supported by the results of the laboratory studies indicated that P2 was an exopeptidase and released free amino acids and possibly di and tri-peptides from the terminal ends of polypetide chains. If this was the case the pretreatment of soya protein with P2 would be analogous to providing free amino acids in the diet. As there is some doubt as to the efficacy of free amino acid supplementation in diets for meal fed pigs it was concluded that the use of P2 was inappropriate in this case.

4.2. The effect of protease pretreatment of raw or autoclaved soyabean meal on the growth performance of liquid fed grower pigs.

4.2.1 *Introduction.*

Although some work has been reported on the effect of pretreatment of soyabean meals with individual proteases on the digestibility and growth performance in newly weaned pigs (Hessing *et al.* 1996; Rooke *et al.* 1996; Caine *et al.* 1997a; Rooke *et al.* 1998) no data is available on the effect of protease treatment of raw soyabean or soyabean meals in liquid diets fed to grower or finisher pigs.

In laboratory studies, reported in section 3.4, pretreatment with protease P4 showed similar increases in the *in vitro* N digestibility (IVDN), with respect to the control (no protease pretreatment), in raw soyabean as P2, (*circa* 7 % and 8 % respectively). This was significantly lower ($P < 0.05$) than the increase obtained with P3, *circa* 10 %. However, pretreatment with P4 reduced trypsin inhibitor activity after *in vitro* peptic digestion from 26.7 to 6.4 mg trypsin inhibited g^{-1} raw soyabean DM compared with 10.6, 16.8 and 12.1 for untreated soya or soya or pretreated with P2 or P3 respectively. P4 also gave the greatest increase in IVDN for autoclaved soyabean meal, 12 % compared with 11 % and 8 % for P2 and P3 respectively. Therefore, P4 was selected for use in this study. The *in vitro* studies indicated that pretreatment of both raw and autoclaved soyabean meal with P4 may have benefits in increasing the utilization of soya protein by the pig. The first part of this study (trial 1a) was conducted to determine if the increase in *in vitro* N digestibility was reflected in increased growth performance in the pig.

One of the pig's requirements for water is to meet the needs of excretion of the products of protein digestion and metabolism and to remove any toxins present in the diet (Brooks and

Carpenter 1990). Therefore, if protein in the diet is not fully utilized or the diet contains toxic substances such as the antinutritional factors present in raw soyabeans this may be reflected in increased water intake. Also, a common observation in studies in which raw soyabean has been fed to grower and finisher pigs has been a reduction in feed intake. (Jiminez *et al.* 1963; Combs *et al.* 1967; Young 1967; Vandergrift *et al.* 1983; Crenshaw and Danielson 1985b). The second part of this study (trial 1b) investigated the effect on water intake and voluntary feed intake of groups of pigs fed diets containing either raw or autoclaved soyabean meals untreated or pretreated with P4. Thus the objectives of this study were to determine:

- whether pretreatment of raw or autoclaved soyabean meal by steeping for 24 h at 20° C in the presence or absence of 20 000 units P4 g^{-1} N increased the growth performance of growing pigs.
- the voluntary feed intake and water requirements of growing pigs fed raw or autoclaved soyabean meal pretreated by steeping for 24 h at 20° C in the presence or absence of 20 000 units P4 g^{-1} N.

4.2.2 *Materials and methods*

Experimental design and trial procedure

Trial 1a

A feeding trial was conducted according to a 2 x 2 factorial design in which the factors were :

1. the soya component of the diet, either raw or autoclaved
2. the enzyme treatment, with or without protease P4.

Thus the four dietary treatments were:

R - Raw soya with no enzyme treatment

A - Autoclaved soya (109°C for 5 min) with no enzyme treatment

R + Raw soya pretreated with 20 000 units protease P4 per gram N

A + Autoclaved soya pretreated with 20 000 units protease P4 per gram N

Thirty two male pigs of similar age and an average weight of 29.2 ± 3.7 kg were randomly allocated to one of the four dietary treatments. The pigs were identified by eartags and housed in pens of four in an environmentally controlled building. Each pen consisted of a kennelled area, a slatted dunging area containing a nipple drinker and four gated feeding stalls (Appendix 4). The pigs were randomly allocated to pens to give one pig on each dietary treatment per pen of four pigs. The pigs were allowed an acclimatisation period of one week during which they were fed dry pelleted feed (Grower 528, BOCM). The pigs were reweighed prior to the commencement of the trial and the average weight was 33.5 ± 4 kg.

During the trial the pigs were fed individually, twice daily, at 09.00 and 16.00 hours, according to a feeding scale. Published figures suggest that feed intake achievable under farm conditions for growing pigs is 2.4 x metabolic body weight (Whittemore 1993). Therefore, to ensure that the allowance was within the voluntary feed intake capacity of the pig a scale was constructed whereby the daily feed offered was calculated as 2.2 x metabolic body weight ($W^{0.63}$) MJ DE d^{-1} (Appendix 5). The pigs were weighed weekly and feeding levels adjusted for each individual pig according to its weight. During the weekly interval between weighings feed offered was increased on a daily basis according to the previous week's average daily weight gain for that pig.

Feed allowances were prepared individually for each pig according to its diet allocation. The pigs were contained in the feeding stalls immediately prior to feeding and allowed to remain in the stalls for 30 minutes to consume their food. Any feed remaining after this period was removed from the trough, weighed back, dried to constant weight in a forced air oven at 90° C and dry matter feed intake calculated. Dry matter feed intake was recorded daily and pig live weight weekly for the six week duration of the trial.

Trial 1b

Sixteen Large White x (Large white x Landrace) male grower pigs weighing 27 ± 4 kg were randomly allocated to four pens and fed each of the diets, (R-, A-, R+, A+) in rotation according to a 4 x 4 Latin Square design. The pigs were identified by eartags housed in an environmentally controlled building (Appendix 4). Each pen consisted of a kennelled area, a slatted dunging area and eight gated feeding stalls of which the pigs had continuous access to four. Each pen contained a nipple drinker with a water supply which was metered (PSML, Kent Meters, Luton). Using a Latin Square design each pen of four pigs was offered one of the experimental diets for a period of 10 days (Table 4.2.1.). Feeds were prepared for each pen according to the diet allocation for that time block. Feed was offered *ad libitum* using four troughs to which the pigs had continous access. Unfinished feed was weighed back on a daily basis, mixed and a representative sample from each trough dried to constant weight in a forced air oven at 90° C to enable dry matter feed intake to be calculated. The pigs were weighed at the start of the trial and at the end of each 10 day block. Water intake per pen was recorded daily.

Table 4.2.1. Latin square design of dietary treatment periods per pen of 4 pigs in Trial 1b.

	diet per 10 day time block			
pen\block	1	2	3	4
1	R -	R +	A -	A +
2	R +	A +	R -	A -
3	A -	R -	A +	R +
4	A +	A -	R +	R -

Diets

Whole raw soya beans (obtained from Parnutt Foods Sleaford, Lincolnshire, UK) from the same batch were used for both raw and autoclaved diet formulations. Autoclaved soya was prepared by spreading whole beans to a depth of 2cm on metal trays and autoclaving at 109° C for 5 minutes. The beans were then allowed to cool and dry. Both raw and autoclaved soyabean were ground by a commercial mill (Fisherbridge Mills, Exeter, UK). The amino acid profile of both the raw and autoclaved soyabeans was determined by Finnfeeds International Ltd and are presented in Table 4.2.2.

The diets were formulated using a feed formulation computer programme (Ultramix-Professional, AGM Systems Ltd, Exeter UK) specifying the amino acid analysis of the appropriate soyabean meal (raw or autoclaved). Soya comprised 30 % of the diet, the remaining 70 % consisting of a basal diet comprising 53.4 % wheat, 7.5 % rapeseed extract, 4.7 % wheatfeed, 2.5 % vitamin and mineral supplement, 1.25 % vegetable oil, 0.6 % dicalcium phosphate and 0.17 % salt. The nutrient analysis of the complete diet is presented in Table 4.2.3.

The diets were formulated to provide 0.71 g lysine MJ DE^{-1} (~12 % less than requirement). Ratios of the other amino acids were adjusted with respect to lysine to

provide amino acids in the proportions necessary to meet the requirements of the pig according to the concept of the ideal protein (Cole 1996).

The feed was prepared by mixing the appropriate amount of soya with water in a ratio of one part soya to three parts water. Protease P4 was added to the soya for diets R + and A + to give an inclusion level of 20 000 units g^{-1} N. The resulting slurries were steeped for 24 hours at 20° C. Immediately prior to feeding the basal diet was added with additional water to give a final water:feed ratio of 3:1.

Table 4.2.2. Crude protein and amino acid analysis of raw and autoclaved soyabeans

	raw soya	autoclaved soya
crude protein %	34.42	34.56
amino acid profile %		
Methionine	0.55	0.52
Cystine	1.04	0.98
Lysine	2.20	2.20
available Lysine	2.11	2.09
Threonine	1.33	1.30
Arginine	2.50	2.49
Isoleucine	1.56	1.55
Valine	1.66	1.67
Histidine	0.91	0.91
Phenylalinine	1.76	1.76
Glycine	1.48	1.48
Serine	1.71	1.70
Proline	1.70	1.75
Alanine	1.50	1.52
Asparagine	3.86	3.84
Glutamine	5.93	5.95
Tyrosine	1.25	1.27

Table 4.2.3. Nutrient analysis of the experimental diets.

nutrient	amount
Dry matter kg	0.88
Digestible energy MJ/kg	14.00
Crude protein %	19.65
Oil %	7.69
Crude fibre %	4.00
Lysine %	1.00
Methionine %	0.30
Methionine + cysteine %	0.55
Threonine %	0.64
Isoleucine %	0.94
Tryptophan %	0.26
Calcium %	1.00
Phosphorus %	0.70
Salt %	0.50
Bulk kg	1.00

Statistical Analysis

For trial 1a. the average daily dry matter feed intake, average daily live weight gain and feed conversion ratios were calculated on a weekly basis. The data were subjected to an analysis of covariance using the weight of the pigs at the start of the trial as the covariate factor. For trial 1b. average daily water intake, average daily dry matter feed intake, average daily live weight gain and feed conversion ratios were calculated for each 10 day block and analysed using a general linear model. All data sets were checked for normal distribution prior to analysis using Minitab version 10.2. (Minitab inc., State College, Pennsylvania USA 1994). Treatment means were compared using Newman- Keuls multiple range test (Zar 1999).

4.2.3. *Results*

Trial 1a.

The factorial analysis of the biological performance of the pigs is summarised in Table 4.2.4. Autoclaved soya significantly increased (P <0.01) overall average daily feed intake by 13 % (0.17, s.e.d. 0.033 kg d^{-1}) and average daily gain (P <0.001) by 40 % (0.23, s.e.d. 0.042 kg d^{-1}) and significantly decreased feed conversion ratios (P<0.001) by 19 % (0.451, s.e.d. 0.043) compared with raw soya. Analysis of the weekly performance data showed these effects were manifest early in the trial and persisted throughout. The addition of P4 significantly increased overall average daily gain (P <0.05) by 11 % (0.07, s.e.d. 0.042 kg d^{-1}) and decreased overall feed conversion ratios (P <0.05) by 5 % (0.103, s.e.d. 0.043). However, these effects only became apparent as the trial progressed and the differences only became significant in the latter part of the trial.

The effect of interactions between the type of soya and presence or absence of the enzyme on the biological performance of the pigs is summarised in Table 4.2.5. For the pigs fed on the raw soya diets the addition of P4 significantly increased (P <0.05) overall average daily feed intake and weight gain by 10 % (0.13, s.e.d. 0.05 kg d^{-1}) and 15 % (0.08, s.e.d. 0.03 kg d^{-1}) respectively. This resulted in a significant (P <0.05) increase in total weight gain of 14 % (3.22, s.e.d. 1.36 kg) due to P4 addition in raw soya diets. However, this was not reflected in comparable significant improvements in feed conversion ratios. Although enzyme treatment of autoclaved soya did show some numerical improvements in daily gain and feed conversion ratios in some weeks these were not statistically significant.

Table 4.2.4. Factorial analysis of adjusted means of growth performance of grower pigs fed diets containing raw or autoclaved soyabean meal steeped for 24 h at 20° C in the presence or absence of 20 000 units g^{-1} N protease P4 with live weight of pigs at the commencement of the trial as a covariate factor.

Parameter	Period	Factor 1: soya		Factor 2 : enzyme		s.e.d.
		Raw	Autoclaved	-	+	
Average Daily Dry Matter Feed Intake (kg pig d^{-1})	week 1	0.69^{d}	0.94^{d}	0.76	0.87	0.082
	week 2	1.03^{x}	1.27^{x}	1.12	1.19	0.059
	week 3	1.35^{x}	1.48^{x}	1.39	1.45	0.032
	week 4	1.49^{d}	1.60^{d}	1.51	1.57	0.031
	week 5	1.59^{x}	1.73^{x}	1.64	1.68	0.024
	week 6	1.69^{x}	1.86^{x}	1.75^{d}	1.80^{d}	0.023
	overall	1.31^{x}	1.48^{x}	1.36	1.43	0.033
Average Daily Gain (kg pig d^{-1})	week 1	0.18^{d}	0.56^{d}	0.29	0.44	0.108
	week 2	0.61^{x}	0.78^{x}	0.69	0.69	0.042
	week 3	0.66^{x}	0.82^{x}	0.74	0.75	0.042
	week 4	0.62^{x}	0.82^{x}	0.69	0.75	0.051
	week 5	0.65^{x}	0.93^{x}	0.77	0.81	0.023
	week 6	0.70^{x}	0.98^{x}	0.77^{x}	0.90^{x}	0.031
	overall	0.57^{x}	0.80^{x}	0.65^{a}	0.72^{a}	0.042
Dry Matter Feed Conversion Ratio	week 1	#	#	#	#	#
	week 2	1.75	1.64	1.67	1.72	0.108
	week 3	2.12^{a}	1.81^{a}	1.91	2.03	0.128
	week 4	2.50^{d}	1.98^{d}	2.31	2.17	0.163
	week 5	2.46^{x}	1.87^{x}	2.20	2.14	0.062
	week 6	2.48^{x}	1.93^{x}	2.37^{d}	2.04^{d}	0.095
	overall	2.31^{x}	1.86^{x}	2.13^{a}	2.03^{a}	0.043

Table 4.2.4 continued..

Parameter	Factor 1: soya		Factor 2 : enzyme		s.e.d.
	Raw	Autoclaved	-	+	
Average Initial Weight (kg)	33.86	32.98	32.51	34.32	1.432
Average Final Weight (kg)	57.28^{x}	67.02^{x}	60.79^{a}	63.51^{a}	0.968
Average Total Gain (kg)	23.83^{x}	33.57^{x}	27.34^{a}	30.06^{a}	0.968

superscripts indicate that means within the same factor in the same row differ significantly
^{a} $P<0.05$; ^{d} $P<0.01$; ^{x} $P<0.001$.
FCR for week 1 was not calculated because four of the pigs on diet R - had negative growth

Table 4.2.5. Performance of pigs fed diets containing raw (R) or autoclaved (A) soyabean meal steeped for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units g^{-1} N P4

Parameter	Period	Diet				s.e.d.
		R -	R +	A -	A +	
Average Daily Dry Matter Feed Intake (kg pig d^{-1})	week 1	0.62^{a}	0.77	0.91	0.96^{a}	0.118
	week 2	0.93^{xfa}	1.13^{a}	1.31^{f}	1.24^{x}	0.079
	week 3	1.30^{xf}	1.41	1.48^{f}	1.49^{x}	0.041
	week 4	1.43^{fgh}	1.54^{h}	1.59^{g}	1.60^{f}	0.050
	week 5	1.55^{xy}	1.63^{fa}	1.73^{ya}	1.74^{xf}	0.031
	week 6	1.66^{xza}	1.73^{ywa}	1.84^{zw}	1.88^{xy}	0.032
	overall	1.24^{xya}	1.37^{a}	1.48^{y}	1.49^{x}	0.049
Average Daily Gain (kg pig d^{-1})	week 1	0.07^{ac}	0.28^{b}	0.51^{c}	0.61^{ab}	0.160
	week 2	0.58^{fb}	0.63^{ac}	0.81^{bc}	0.75^{fa}	0.062
	week 3	0.65^{ac}	0.67^{bd}	0.82^{cd}	0.82^{ab}	0.059
	week 4	0.58^{fb}	0.66^{a}	0.80^{b}	0.85^{fa}	0.071
	week 5	0.63^{xz}	0.67^{yw}	0.91^{zw}	0.96^{xy}	0.031
	week 6	0.63^{xzc}	0.77^{ybc}	0.91^{azb}	1.04^{xya}	0.052
	overall	0.53^{xza}	0.61^{ywa}	0.77^{zw}	0.83^{xy}	0.033
Dry Matter Feed Conversion Ratio	week 1	#	#	#	#	#
	week 2	1.688	1.816	1.642	1.630	0.152
	week 3	2.012	2.232	1.808	1.819	0.180
	week 4	2.555^{a}	2.437	2.054	1.901^{a}	0.231
	week 5	2.470^{xz}	2.452^{yw}	1.919^{zw}	1.822^{xy}	0.103
	week 6	2.699^{xa}	2.267^{y}	2.049^{xfa}	1.812^{xyf}	0.135
	overall	2.353^{xz}	2.262^{yw}	1.915^{zw}	1.799^{xy}	0.061

Table 4.2.5 continued

Parameter	Period	Diet				s.e.d.
		R -	R +	A -	A +	
Average Initial Weight (kg)		32.91	34.81	32.11	33.84	2.018
Average Final Weight (kg)		55.67^{xza}	58.89^{ywa}	65.91^{zw}	68.13^{xy}	1.363
Average Total Gain (kg)		22.22^{xza}	25.44^{ywa}	32.46^{zw}	34.68^{xy}	1.363

means in the same row having the same superscript differ from each other
^{a,b,c,d} $P<0.05$; ^{f,g} $P<0.01$ ^{w,x,y,z} $P<0.001$
FCR for week 1 was not calculated because four of the pigs on diet R - had negative growth

It was observed that many of the pigs on the raw soya diets had a very much reduced feed intake for the first 5 - 7 days of the trial which resulted in four of them losing weight over the first week. The data also showed that there was a considerable variation in feed intake and weight gain over the group of 32 pigs as a whole. The variation in feed intake was particularly noticeable in the pigs on diet R -. The data presented in Figure 4.2.1.a.-b. shows the range of daily weight gain and daily feed intake observed for pigs on each diet. Frequency histograms of average daily feed intake (Fig. 4.2.2.a) and average daily weight gain (Fig. 4.2.2.b.) using intervals of 0.025 kg illustrate the distribution of data for these two parameters.

The growth rate of pigs fed RSB diets was significantly lower (ADG 0.57 kg pig^{-1} d^{-1}) than that of the pigs fed ASB diets (ADG 0.80 kg pig^{-1} d^{-1} s.e.m. 0.02). The poor feed intake and consequently low or negative weight gain of some of the pigs fed RSB diets resulted in them being placed on a low point on the feed scale initially. There was some concern that this could have had a cumulative effect which resulted in a low growth rate throughout the trial. In order to ascertain if this was the case the growth curves of individual pigs from day 7 - 42 (i.e. from the time feed refusals ceased) were plotted. These data are presented in Figures 4.2.3 and 4.2.4.a.-d. The slopes of the growth curves were subjected to analysis of covariance, with the weight of the pigs at the start of trial as the covariate factor. This analysis is presented in Table 4.2.6. The growth rate of pigs fed autoclaved soya was significantly increased ($P < 0.001$) by 0.21 (s.e.d. 0.02) kg d^{-1} compared with the pigs fed diets containing raw soya. However, when the interaction between soyabean meal and P4 addition was analysed there was a significant increase of 0.06 (s.e.d. 0.03) kg d^{-1} for pigs fed raw soya diets but no significant effect on pigs fed autoclaved diets due to P4 addition. These figures were slightly different than those

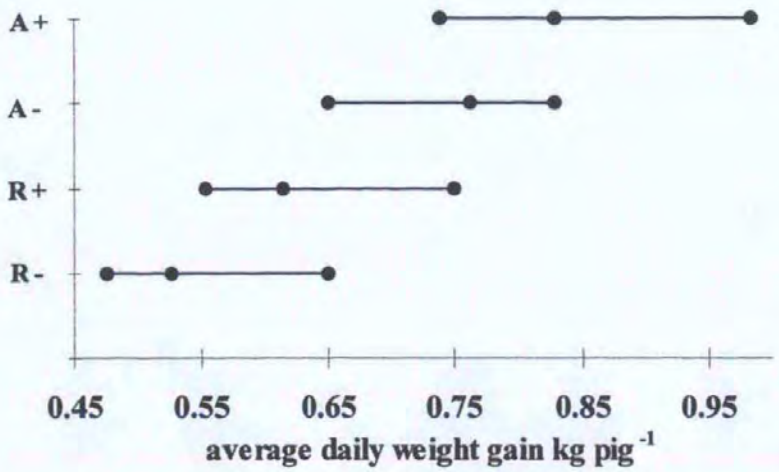


Figure 4.2.1.a Range of daily weight gain of pigs fed diets containing raw soyabean (R) or autoclaved soyabean (A) pretreated by steeping for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units P4 g^{-1} N

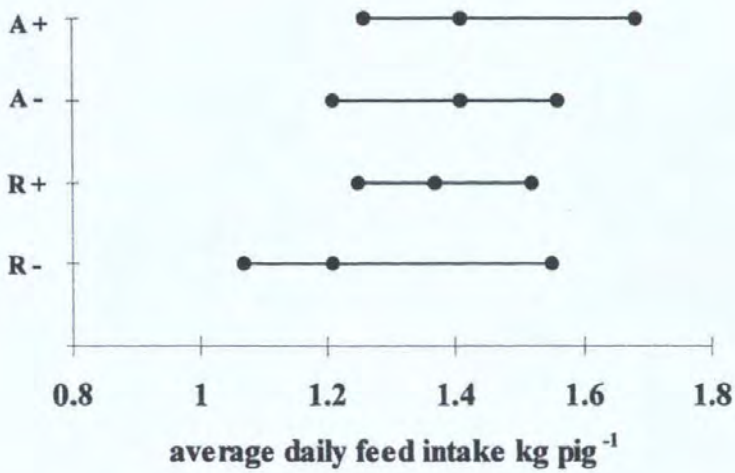


Figure 4.2.1b Range of daily feed intake of pigs fed diets containing raw soyabean (R) or autoclaved soyabean (A) pretreated by steeping for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units P4 g^{-1} N

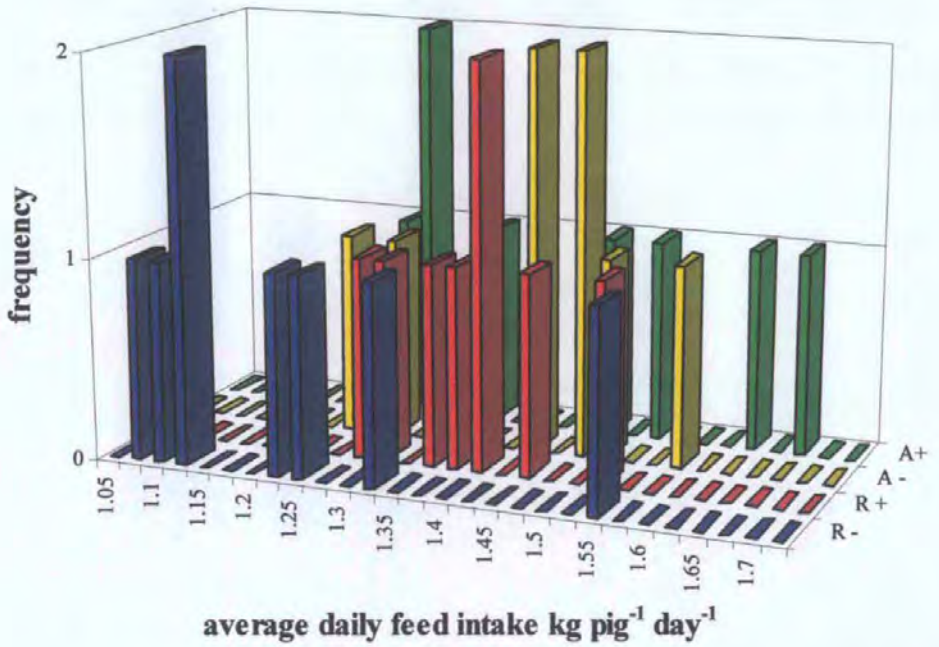


Figure 4.2.2.a Frequency histogram of average daily feed intake of pigs fed diets containing raw soyabean (R) or autoclaved soyabean (A) pretreated by steeping for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units P4 g^{-1} N

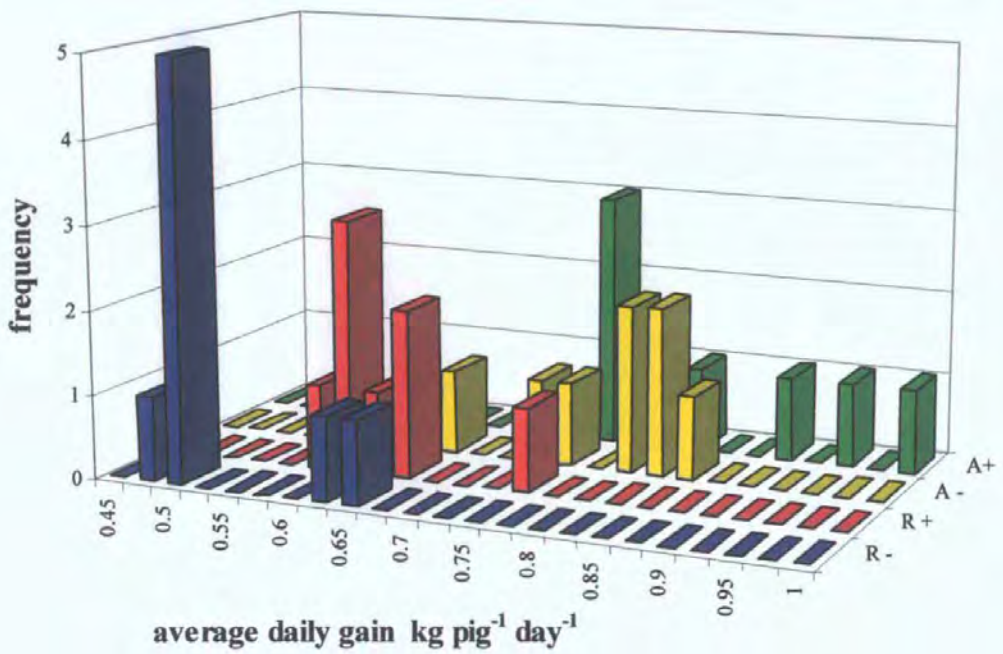


Figure 4.2.2.b Frequency histogram of average daily gain of pigs fed diets containing raw soyabean (R) or autoclaved soyabean (A) pretreated by steeping for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units P4 g^{-1} N

Table 4.2.6. Factorial analysis of the growth rate of pigs (kg live weight gain d^{-1}) fed liquid diets containing raw or autoclaved soyabean meal steeped for 24 h at 20° C in the presence or absence of 20 000 units g^{-1} N P4

	Soyabean meal			
Protease P4 (units g^{-1} N)	Raw	Autoclaved	Main effect: protease	s.e.d.
0	0.62^{x1}	0.83^{x}	0.73^{1}	0.020
20 000	0.68^{x1}	0.88^{x}	0.78^{1}	
Main effect: soya	0.65^{x}	0.86^{x}		
s.e.d.	0.020			

[1] means with the same superscript in the same column differ significnatly ($P < 0.05$)
[x]means with the same superscript in the same row differ significantly ($P < 0.001$)
s.e.d. for the interaction between soyabean meal and protease = 0.032

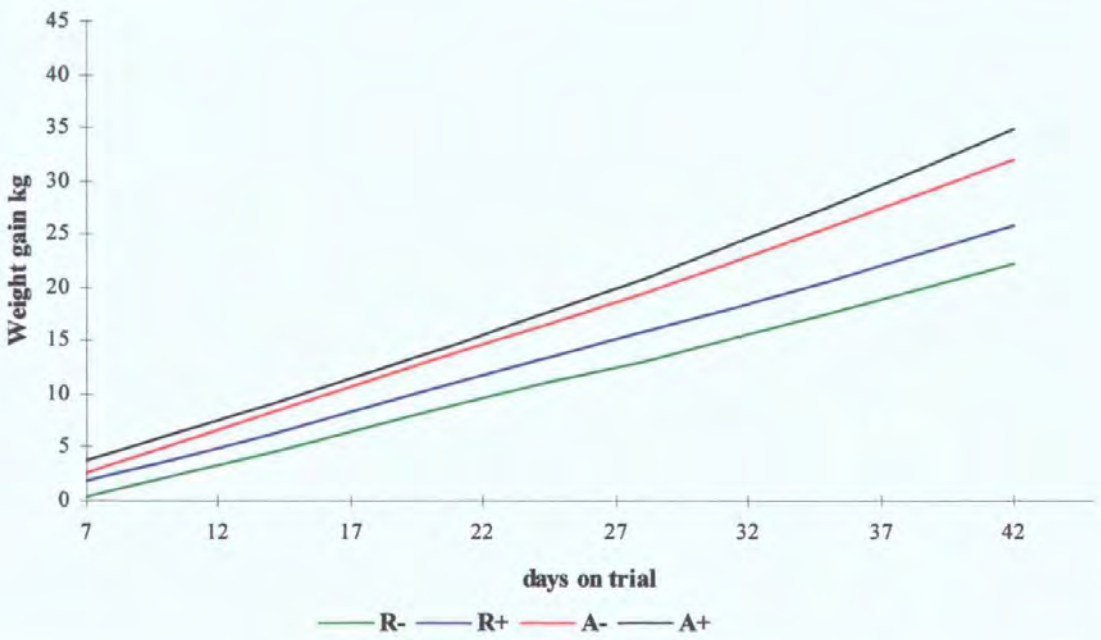
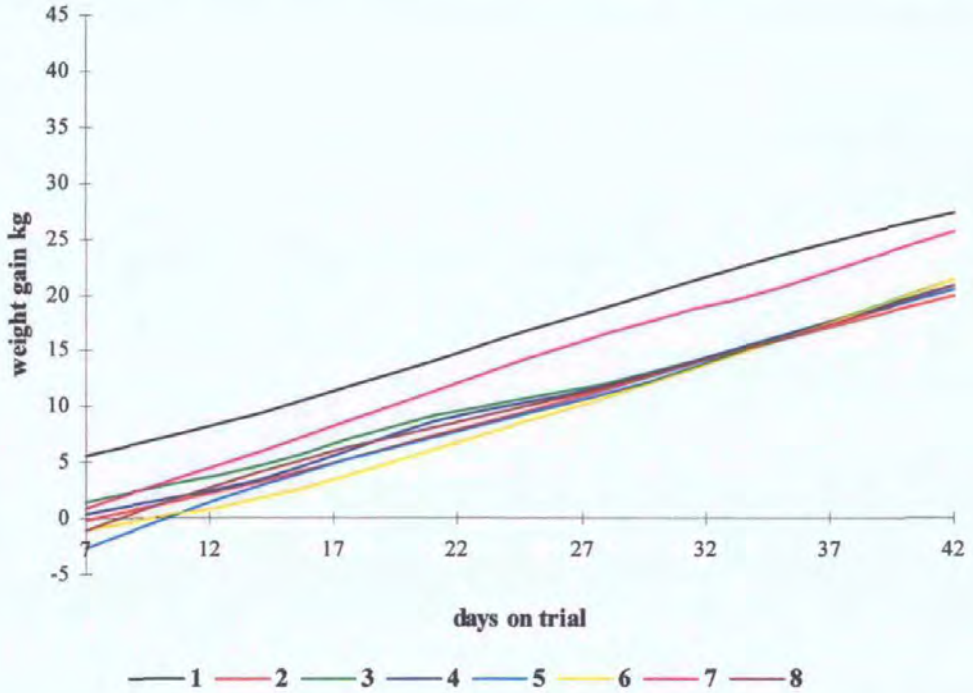
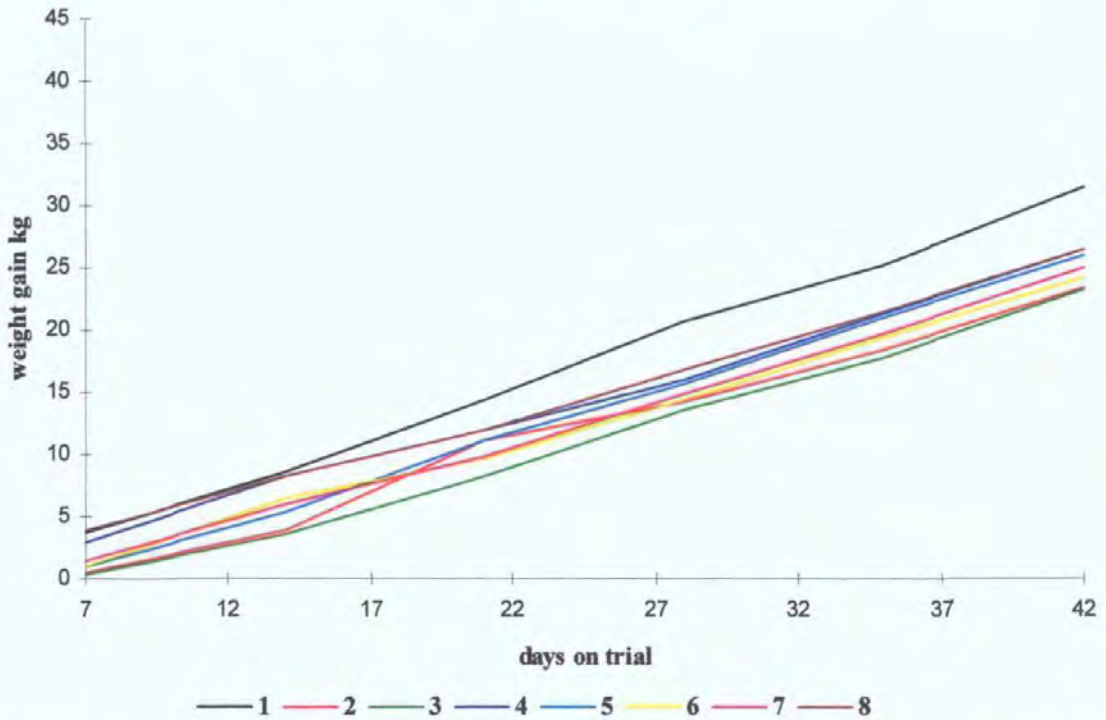


Figure 4.2.3. Mean growth rate of pigs (from day 7) fed diets containing raw (R) or autoclaved (A) soyabean meal steeped for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units g^{-1} N P4.

Figure 4.2.4. Growth rates (from day 7) of individual pigs on each dietary treatment

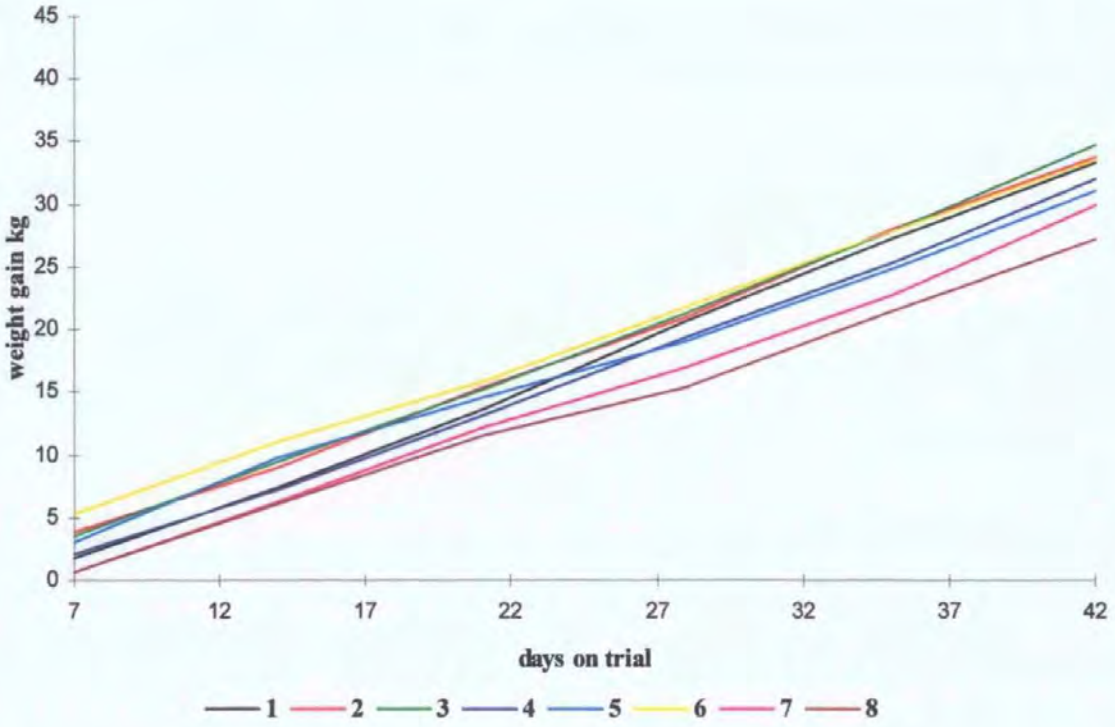


a) Growth rates of individual pigs fed diets containing raw soyabean meal steeped for 24 h at 20° C in the absence of protease P4. (R-)

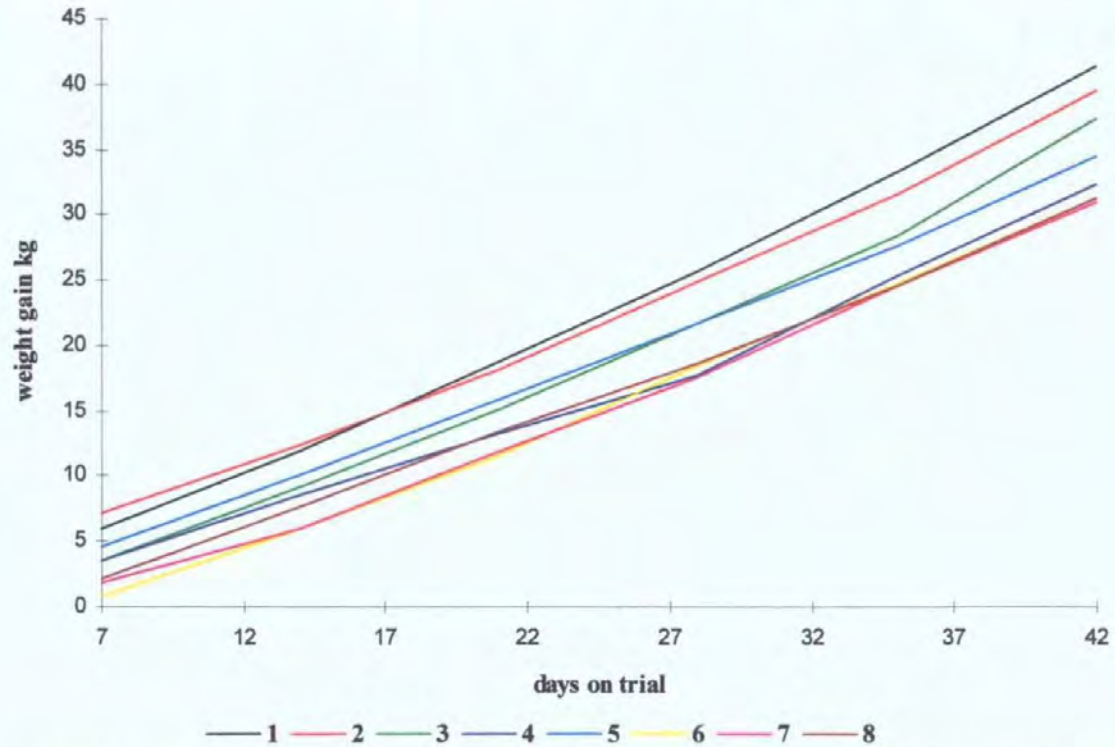


b) Growth rates of individual pigs fed diets containing raw soyabean meal steeped for 24 h at 20° C in the presence of 20 000 units g^{-1} N of P4. (R+)

inercept on Y axis indicates weight gain / loss in the first 7 d.



c) Growth rates of individual pigs fed diets containing autoclaved soyabean meal steeped for 24 h at 20° C in the absence of P4. (A-)



d) Growth rates of individual pigs fed diets containing autoclaved soyabean meal steeped for 24 h at 20° C in the presence of 20 000 units g^{-1} N P4. (A+)

inercept on Y axis indicates weight gain / loss in the first 7 d

obtained for overall ADG (Table 4.2.5) because they were calculated from day 7 to day 14 rather than the whole period of the trial. Although there appeared to be some variation in the slopes of the growth curves for individual pigs on each dietary treatment, when the slopes of the regression lines were compared there was no statistically significant difference between them.

Trial 1b

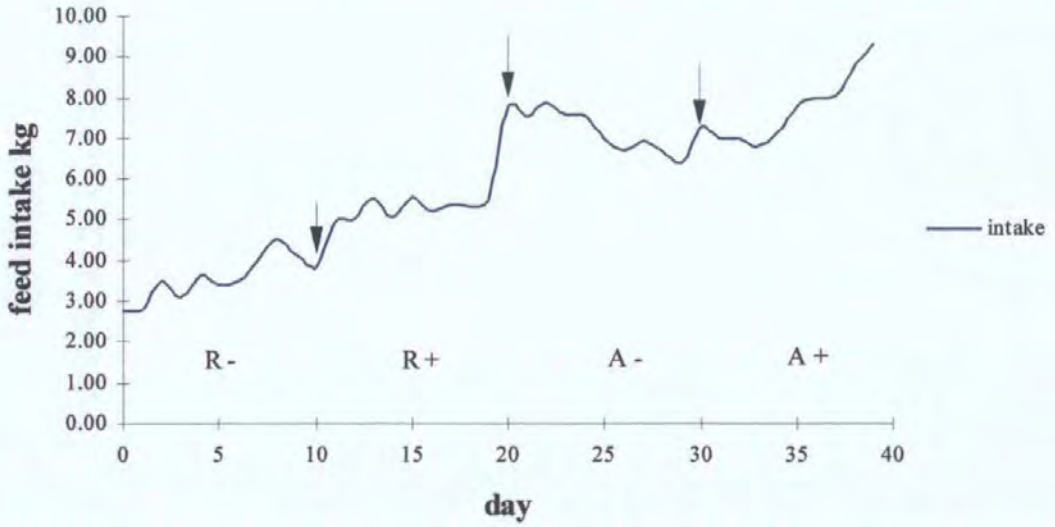
The biological performance of the pigs on trial is summarised in Table 4.2.7. Analysis of variance for these data using a general linear model showed that the most significant factor affecting feed intake was the time period. However, the most significant factor affecting average daily gain and feed conversion ratio was the diet. Diet appeared to have no effect on water intake. However, water intake may have been affected by the fact that during the trial the weather was particularly hot and during the day the temperature in the building often rose above 25° C. As with Trial 1a it was observed that inclusion of raw soyabean in the diet reduced feed intake in these pigs. This effect was particularly noticeable when the pigs changed from one of the autoclaved soya diets to one of the raw soya diets. Data on changes in daily feed intake over time are presented in Figures 4.2.5.a.-d.

Table 4.2.7. Growth performance and water intake for groups of four pigs fed *ad libitum* diets containing raw (R) or autoclaved (A) soyabean steeped for 24 h in the presence (+) or absence (-) of 20 000 units P4 g^{-1} N.

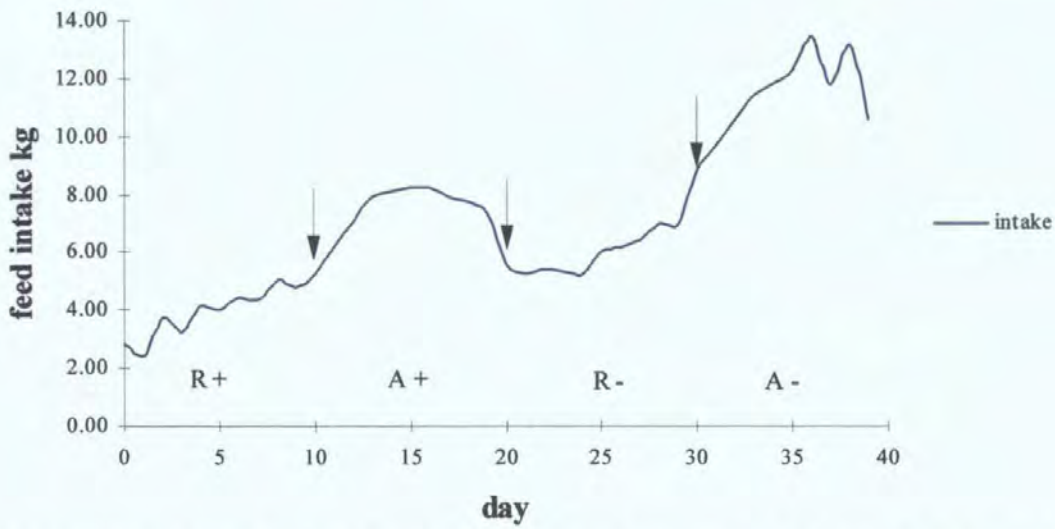
Parameter	Period 1	2	3	4	s.e.d.
Average daily dry matter intake (kg pig^{-1})	1.14[a]	1.60	1.84	2.27[a]	0.203
Average daily gain (kg pig^{-1})	0.64	0.65	0.79	1.01	0.140
Dry matter feed conversion ratio	1.91	2.86	2.5	2.38	0.295
Average daily water intake (litres kg^{-1} DMFI)	8.3	9.2	11.6	12.0	2.360
	Diet R -	R +	A -	A +	
Average daily dry matter intake (kg pig^{-1})	1.55	1.46	1.98	1.86	0.203
Average daily gain (kg pig^{-1})	0.50[a]	0.55[b]	1.05[ab]	0.99	0.140
Dry matter feed conversion ratio	3.26[ab]	2.63	1.90[b]	1.87[a]	0.295
Average daily water intake (litres kg^{-1} DMFI)	10.9	11.7	10.0	8.6	2.360
	Pen 1	2	3	4	
Average daily dry matter intake (kg pig^{-1})	1.48	1.79	1.69	1.89	0.203
Average daily gain (kg pig^{-1})	0.66	0.86	0.76	0.81	0.140
Dry matter feed conversion ratio	2.32	2.28	2.70	2.35	0.295
Average daily water intake (litres kg^{-1} DMFI)	11.9	10.3	11.4	7.5	2.360

Values in the same row with the same superscript differ significantly from each other. a,b, $P < 0.05$

Figure 4.2.5. Pattern of daily feed intake over four 10 day feeding blocks for pigs fed *ad libitum* diets containing raw (R) or autoclaved (A) soya steeped for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units P4 g^{-1} N.



a) Changes in daily feed intake for pigs in pen 1 over four 10 day feed intervals, arrows indicate diet changes



b) Changes in daily feed intake for pigs in pen 2 over four 10 day feed intervals, arrows indicate diet changes.

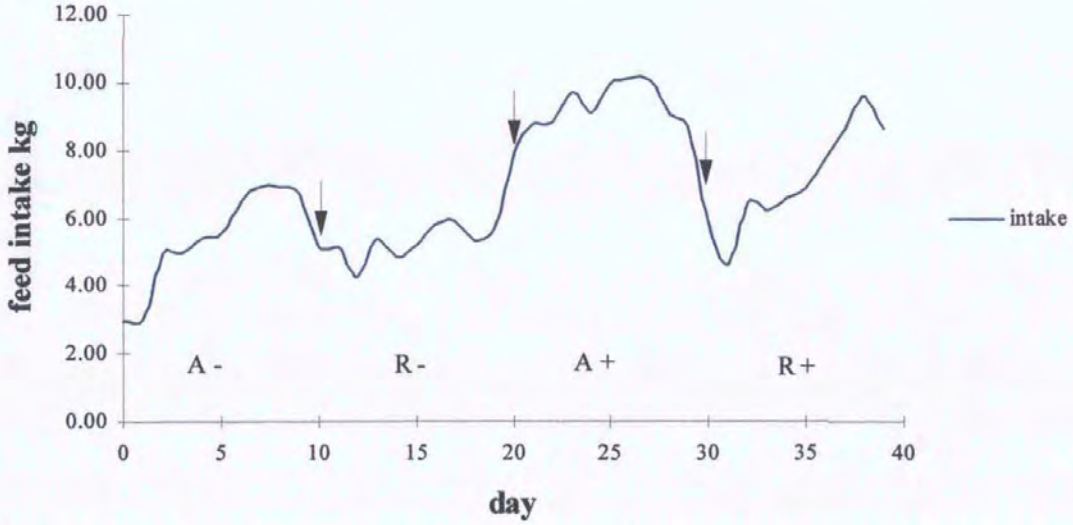
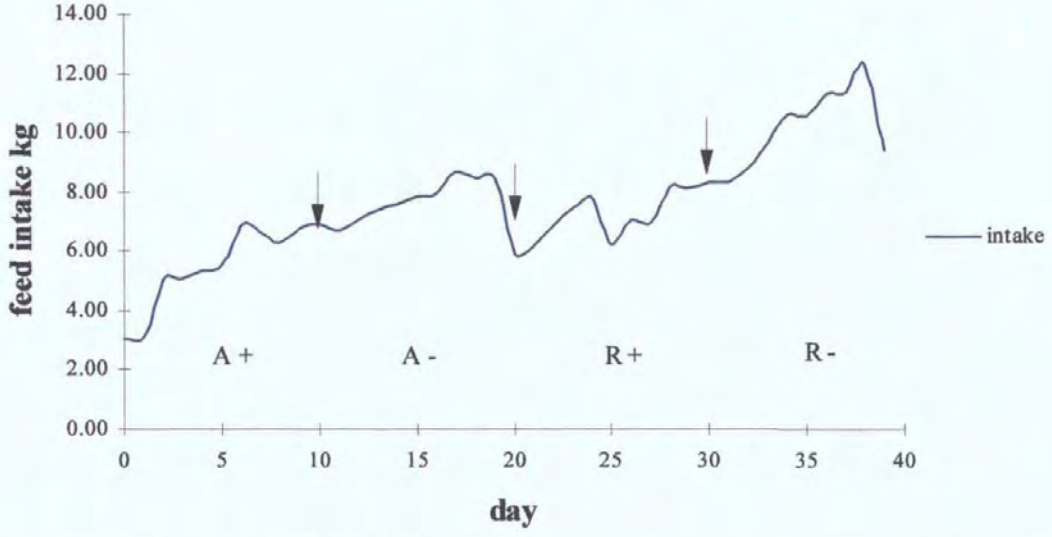


Figure c) Changes in daily feed intake for pigs in pen 3 over four 10 day feed intervals, arrows indicate diet changes.



d) Changes in daily feed intake for pigs in pen 4 over four 10 day feed intervals, arrows indicate diet changes.

4.2.4 *Discussion*

In Trial 1a factorial analysis of the data show that the form of soyabean in the diet has the largest effect on the performance parameters. This is in agreement with the findings of other studies in which inclusion of raw soyabean into diets has been compared with heat treated soyabean (Jiminez *et al.* 1963; Combs *et al.* 1967; Young 1967; Vandergrift *et al.* 1983; Crenshaw and Danielson 1985b). Protease addition appeared to have an effect on average daily gain and feed conversion ratio in the latter part of the trial which contributed to an overall effect on these parameters. When the data for individual diets was analysed the situation was less clear. Significant ($P<0.001$) differences were apparent for all the performance parameters between the forms of soyabean used. The pigs fed diets containing autoclaved soyabean gained on average 9.75 kg more and feed conversion was 0.45 less than the pigs fed raw soya diets over the duration of the trial. However, the overall effect of protease addition appeared to be due mainly to its effect on raw soyabean diets. Feed intake in the raw soyabean diets was significantly increased ($P <0.05$) from week 2 onwards by protease addition, whereas there was no difference in feed intake for either of the autoclaved soya diets. The initial reluctance of the pigs to consume the raw soya diets suggested that the reduction in feed intake was at least in part due to poor palatability of these diets. The data presented in Figures 4.2.1.b. and 4.2.2.a. show that the addition of P4 reduced the variability of feed intake in pigs fed diets containing raw soyabean. Therefore, it could be speculated that partial hydrolysis of protein in raw soyabean affected the palatability, making it more acceptable to the pig. The differences in feed intake due to protease addition in the raw soyabean diets were not reflected in increases in daily gain or feed conversion ratios. There was no significant difference in daily gain in either the raw or autoclaved soyabean diets due to protease addition until week 6. In week 6 daily gain increased in pigs fed raw and autoclaved soya diets in response to protease addition. This was reflected in a significant ($P <0.05$) increase in total gain in the pigs fed raw soyabean

diets but not in the pigs fed autoclaved soyabean diets. There were no significant difference in FCR due to protease addition apart from the raw soyabean diets in week 6. Crenshaw and Danielson (1985b) and Cook *et al.* (1988) suggested that tolerance to dietary raw soyabean may be age dependant, with detrimental effects lessening with age. If this is the case the enzyme treatment could have increased utilization of soya protein in the latter part of the trial as the pigs overcame the adverse effects of raw soyabean.

Jiminez *et al.* (1963) and Crenshaw and Danielson (1985b) observed substantial variation between individual pig's responses to raw soyabean. Jiminez *et al.* (1963) reported weight gains ranging from 16 - 62 kg and 19 - 51 kg for grower pigs fed raw soyabean over a 12 week trial period and Crenshaw and Danielson (1985b) reported weight gains ranging from 8 - 65 kg over a 14 week trial period. In this study the range of total weight gains did not appear to be so substantial and there was little difference between them due to dietary treatment. Total weight gain ranged from 20.0 - 27.3 kg, 23.2 - 31.5 kg, 27.3 - 34.8 kg and 31.0 - 41.3 kg for diets R -, R +, A - and A + respectively. The data presented in Figure 4.2.2. showed there to be considerable variation in feed intake with all diets, with some of the pigs on the raw soyabean diets consuming as much and in some cases more than pigs on the autoclaved soya diets. This was not, however, reflected in daily gain as most of the pigs fed raw soyabean gained less (651 ± 16 g d^{-1}) than the pigs fed autoclaved soyabean (716 ± 16 g d^{-1}), with the exception of one pig on the P4 treated raw soyabean whose daily gain (793 g d^{-1}) was equal to that of some of the pigs fed autoclaved soyabean (range 650-983 g d^{-1}).

Although there was some concern that the low feed intake, and consequent low weight gain, of some of the pigs during the first 7 days of the trial resulted in them being compromised throughout the trial due to being allocated low feed allowances, this appeared

to be unfounded. There appeared to be a large variability in the growth rate of pigs on some of the diets, notably diets R+ and A+, in which the growth curves appeared to diverge. However, the statistical analysis of the growth curves of individual pigs showed no significant differences in the slopes of the growth curves of individual pigs on each diet. In comparing the slopes, the statistical test took into account the residual standard deviation for each line of best fit, which may account for the lack of statistical significance between them. Although the growth rates on each diet were the same the overall weight gain of pigs that lost weight in the first 7 d was reduced due to the weight loss incurred at the start of the trial.

Although P4 treatment appeared to have some beneficial effect on growth performance overall the increases seen in the *in vitro* studies were not reflected in increased growth performance in the pig. The reduction in trypsin inhibitor levels after *in vitro* peptic digestion due to pretreatment with P4 observed in the laboratory studies (section 3.5.3) did not appear to be sufficient to overcome their growth depressing effect in the pig.

The results for Trial 1b appear somewhat contradictory. The diet had no statistcally significant effect on feed intake overall, although it was observed that intake was reduced when the pigs were fed either of the raw soyabean diets. Figure 3a - d shows the pattern of feed intake over the duration of the trial. It is quite apparent from the charts for pen 2 (Figure 4.2.4.b.) and pen 3 (Figure 4.2.4.c.) that feed intake fell when the pigs changed over from an autoclaved soyabean diet to a raw soyabean diet. The reduction in feed intake was quite obvious during the trial with much of the feed being left in the troughs for the few days after a changeover from an autoclaved to a raw soya diet. The reduction in feed intake was reflected in reduction in average daily gain which was approximately 50 % lower in pigs on the raw soya diets. This supported the observation from Trial 1a, that

pigs found raw soyabean unpalatable and that this contributed to a reduction in feed intake. The opposite effect was observed when pigs changed from a raw soyabean diet to an autoclaved soyabean diet. In this study a Latin Square experimental design was used. Inherent in this design was the supposition that individual treatments would have no consequential effects on subsequent treatments. In this case pigs showed a marked increase in feed intake of an autoclaved soya diet if preceded by a raw soya diet, but not if preceded by an autoclaved soya diet. The poor palatability of raw soya diets may have resulted in a lack of satiety due to reduced gut fill. The increased feed intake on changing to a more palatable autoclaved soya diet may have been to satisfy this demand. However, Crenshaw and Danielson (1985b) reported compensatory increases in the performance of pigs fed diets containing soyabean meal for 14 d after previously being fed diets containing raw soya for 98 d. Also, catch up growth and improved nitrogen retention has been observed in pigs after periods of food and nitrogen deprivation (Whittemore, Tullis and Hastie 1978). It is possible that this could have been occurring in these pigs, whereby reduced feed intake and poor utilisation of raw soya protein resulted in nitrogen deprivation, which was followed by a catch up growth when they changed to a more utilizable diet. Therefore, a criticism of the design of this trial must be that it did not measure the factors which it was intended to. The pigs obviously took several days to acclimatize to each dietary change and therefore the short length of time period on each diet prevented accurate measurement of voluntary feed intake. Furthermore, the acclimatization rates for each diet differed and this gave a disproportionate effect on the outcome of the trial as it was conducted over such a short time period. It was considered that the Latin Square design would be unsuitable for subsequent feed trials because of the confounding effects of catch up growth and the possible age related effects of the pigs response to the inclusion of RSB in the diet.

The pigs in pen 4 that received raw soyabean diets in the last two feeding blocks appeared to cope better with these diets than other groups of pigs, although feed intake did fall initially. The pigs in pen 3 also showed an increase in feed intake after an initial reduction on diet R + in the last feeding block. Again these observations may be due to an age related increase in tolerance to raw soyabean.

The dietary treatment appeared to have no significant effect on water intake. However, the metered water intake for each pen of pigs may not represent true water intake due to dietary treatment. A spell of hot weather during the trial led to temperature in the building rising to 30° C on some days, also the pigs were observed to 'play' with the drinkers on numerous occasions, which resulted in inaccurate recordings of water intake.

4.3 The effect of protease pretreatment of raw or micronized soyabean meal on the growth performance and carcass composition of liquid fed grower and finisher pigs.

4.3.1. *Introduction.*

In the previous feeding trial (1a) reported in section 4.2, pretreatment of raw and autoclaved soyabean meal with protease P4 resulted in little overall benefit in increased growth performance of pigs over the six week trial period. In the present study the time period of the feeding trial was extended to cover both the growing and finishing periods and take the pigs to slaughter weight. Micronized soyabean meal (MSB) was used in this study instead of autoclaved soyabean meal because it was not possible to autoclave a sufficient quantity of soyabean to conduct a trial of this duration. P3 was selected to pretreat RSB and MSB because the laboratory studies reported in chapter 3 showed that P3 and P4 gave similar overall percentage increases in *in vitro* nitrogen digestibility (IVDN) of *circa* 7 % relative to the control samples (no protease pretreatment). However, P3 gave a significantly greater increase in IVDN of 10 % *v* 8 % (s.e.m. 1.0) and 8 % *v* 3 % (s.e.m. 1.0) for RSB and MSB respectively. Carcass composition, in terms of the percentage of lean meat, can be estimated from the backfat depth and loin eye muscle depth at the last rib. If the addition of protease pretreated soyabean meal to the diet increased the utilization of protein, this should result in an increase in lean tissue deposition which will be reflected in the ratio of lean to fat tissue in the loin area of the carcass.

Pancreatic hypertrophy has been reported in rats and chicks in response to the inclusion of RSB in the diet but not in pigs (Huisman 1989). It has been postulated that species in which the ratio of pancreas to body weight is less than 0.3% are more susceptible to pancreatic hypersecretion and hypertrophy in response to SPI in the diet than species in which pancreas : body weight ratio exceeds 0.3%. (Liener and Kakade 1980). Pigs fall into

the latter category and data published to date (Yen *et al.* 1977; Schulze *et al.* 1993b) indicates that pigs do not suffer from pancreatic hypertrophy due to consumption of RSB or isolated KTI. However, these studies have been conducted for limited periods of time, 35 d and 10 d respectively. There are no data available on the effects of long term consumption of RSB on pancreas size in pigs.

The objectives of this study were to determine the effect of pretreatment of raw or micronized soyabean meal by steeping for 24 h at 20° C in the presence or absence of 20 000 units P3 g^{-1} N on:

- the growth performance of growing and finishing pigs
- the carcass composition of pigs

4.3.2. *Materials and Methods.*

Experimental Design and trial procedure.

A feeding trial was conducted according to a two factor factorial design in which the factors were:

1. the soya component of the diet, either raw or micronized

2. the enzyme treatment, the presence or absence of protease P3.

Thus the four dietary treatments were:

R - Raw soyabean with no enzyme treatment

M - Micronized soyabean meal with no enzyme treatment

R + Raw soyabean pretreated with 20 000 units P3 g^{-1} N

M + Micronized soyabean meal pretreated with 20 000 units P3 g^{-1} N

Thirty two male pigs of 10 - 11 weeks of age with an average weight of 20.2 ± 2 kg were randomly allocated to one of four dietary treatments. Individual pigs were identified by

eartags and housed in pens of eight in an environmentally controlled building. Each pen consisted of a kennelled area, slatted dunging area containing a nipple drinker (Arato, Bernard partridge, Weeley Heath, Essex) and eight gated feeding stalls. The pigs were randomly allocated to pens to give two pigs per diet per pen. The pigs were allowed a ten day acclimatization period in which they were fed a dry pelleted diet (Grower 528, BOCM). The pigs were reweighed prior to commencement of the trial at which time their average weight was 27.7 ± 3.2 kg.

During the trial the pigs were fed individually, twice daily, at 08.30 and 17.00 hours, according to a feeding scale. To restrict the feed intake of the pigs fed MSB diets to that of the pigs fed RSB diets, the feeding scale was calculated from the average *ad libitum* voluntary feed intake achieved in Trial 1b of pigs offered both RSB diets (Appendix 5). Thus in this trial feed offered equated to 2.0 x metabolic body weight ($W^{0.63}$) MJ DE d^{-1}. The pigs were weighed weekly and the feeding level for each pig was adjusted according to its weight. During each week the quantity of feed was adjusted on a daily basis according to the calculated average daily weight gain from the previous week for each individual pig. Feed allowances were prepared individually for each pig according to its diet allocation. The pigs were confined in the feeding stalls immediately prior to each feed and allowed to remain in the stalls for 30 min. to consume their food. Any food remaining after this time was removed from the trough, weighed, dried to constant weight in a forced air oven at 90° C and dry matter feed intake calculated. Dry matter feed intake was recorded daily and live weight was recorded weekly for the duration of the trial.

The pigs were fed a grower diet until they attained 50 kg liveweight. From 50 kg until slaughter they were fed a finisher diet. Five days after their liveweight exceeded 85 kg for the pigs fed MSB diets and 75 kg for the pigs fed RSB diets the pigs were slaughtered at a

local Abattoir (Gages Abattoir, Ashburton). On the morning of slaughter the pigs were weighed but received no feed. Pigs were stunned and slaughtered by exsanguination. Liver and pancreas weights were recorded on the day of slaughter and cold empty carcass weights were recorded 24 h after slaughter. It was intended for the carcasses to be cut at the 10th rib and percentage lean calculated by measuring P_2 backfat and loin eye muscle depth. Unfortunately, due to a misunderstanding at the slaughterhouse the carcasses were cut through at the 6th rib. Therefore lean and fat areas of the loin were traced onto transparent acetate sheets and the relative areas of lean and fat measured using an area meter (Area Meter mk2, Delta T devices, Cambridge) and the ratio between the two calculated.

Diets

Whole raw soyabeans and full fat micronized soyabean (obtained from Charnwood Milling Company Ltd., Framlingham, Suffolk) from the same batch were used in all diet formulations. Raw soyabeans were ground (Fisherbridge Mills Exeter) prior to inclusion in the diets. A grower and a finisher diet were formulated (Ultramix- Professional, AGM Systems Ltd., Exeter) to provide 0.71 g lysine MJ DE^{-1} and 0.63 g lysine MJ DE^{-1} respectively. The composition of the diets and nutrient specification are presented in Table 4.3.1 and Table 4.3.2 respectively. When formulating the diets the assumption was made that the micronization process did not alter the composition of the soyabean meal.

The feed for individual pigs was prepared by mixing the appropriate quantity of soyabean meal with water in a ratio of one part soya to three part's water and adding 20 000 units of P3 g^{-1} N to diets R + and M +. The resulting slurries were steeped for 24 h and the appropriate quantity of basal diet added immediately prior to feeding along with sufficient water to maintain a feed:water ratio of 1 : 3.

Table 4.3.1 Composition of the experimental diets.

Ingredient	Inclusion level (g kg^{-1})	
	Grower	Finisher
Full fat soya	318	260
	Basal Diet	
wheat	564	369
wheatfeed	77	
barley		326
Vitamin Premix	25	25
Dicalcium phosphate	7.7	7.6
vegetable oil	5.8	
Salt	1.7	5.0
Limestone		0.4

Table 4.3.2. Nutrient analysis of the experimental diets

Nutrient	Grower	Finisher
Dry Matter (kg)	0.88	0.87
Digestible energy (MJ kg^{-1})	14.00	13.50
Crude Protein %	18.90	16.60
Oil %	7.52	5.76
Crude Fibre %	3.40	3.48
Lysine %	0.99	0.85
Methionine %	0.28	0.26
Methionine + Cystine %	0.60	0.55
Threonine %	0.69	0.60
Isoleucine %	0.93	0.81
Tryptophan %	0.24	0.22
Calcium %	1.00	1.00
Phosphorus %	0.70	0.65
Salt %	0.50	0.82

Statistical analysis

Average daily feed intake, average daily gain and feed conversion ratios were calculated for the grower and finisher period and for the overall trial. The results were subjected to analysis of covariance using the weight at the start of the trial as a covariate factor. The killing out percentage, liver:carcass weight, pancreas:carcass weight and lean:fat ratios were calculated and subjected to analysis of covariance. Age at slaughter was used as a covariate factor for killing out %, liver:carcass weight and pancreas:carcass weight, age at slaughter and carcass weight were used as covariate factors for lean:fat. All data were checked for normal distribution prior to statistical analyses. Liver:carcass weight and pancreas:carcass weight data were binomially distributed and were, therefore, transformed prior to analysis using the following transformation:

$$X' = 2\arcsin\sqrt{X} \qquad \text{equation 6}$$

All statistical analyses were undertaken using Minitab (release 10.2, State College, Pennsylvania). Treatment means were compared using Newman-Keuls multiple range test (Zar 1999).

4.3.3. *Results*

The biological performance of the pigs for the grower, finisher and total trial periods are summarized in Tables 4.3.3. and 4.3.4. The factorial analysis (Table 4.3.3.) showed that the factor having the greatest effect on biological performance was the type of soyabean meal. The average daily gain of pigs fed diets containing MSB increased significantly ($P < 0.001$) by 260 g d^{-1} (s.e.d.18) and 300 g d^{-1} (s.e.d. 30) for the grower and finisher periods respectively compared with pigs fed RSB. Although, there was no significant difference in average daily feed intake in the grower period, this was increased by 120 g d^{-1} (s.e.d. 16) for pigs fed MSB compared with RSB in the finisher period. Feed conversion

ratios reflected the increases in ADG, with an improvement in feed conversion of 1.102 (s.e.d. 0.106) and 1.04 (s.e.d. 0.134) for pigs fed MSB compared with RSB in the grower and finisher periods respectively. Average daily gain and feed conversion was consistently better for pigs fed MSB diets than RSB diets throughout the duration of the trial resulting in a significant ($P < 0.001$) overall improvement in ADG of 300 g pig^{-1} d^{-1} (s.e.d. 16) and FCR of 1.04 (s.e.d. 0.088).

Pretreatment of soya with P3 affected pig performance in the finisher period only, significantly ($P < 0.05$) improving ADG by 80 g pig^{-1} d^{-1} (s.e.d.30) and FCR by 0.31 (s.e.d. 0.134) during this period. This resulted in a significant ($P < 0.05$) overall improvement in ADG and FCR of 40 g pig^{-1} d^{-1} (s.e.d. 16) and 0.19 (s.e.d. 0.088) respectively. As with the previous study (Trial 1a) when the interactions between soyabean meal and protease pretreatment were analysed (Table 4.3.4.) the effect of P3 addition was most apparent in the RSB diets. Protease pretreatment of RSB significantly increased ($P < 0.05$) ADG by 100 g d^{-1} (s.e.d. 41) and improved FCR by 0.476 (s.e.d. 0.19) in the finisher period. The addition of P3 to MSB diets showed some numerical improvements in performance parameters throughout the trial but these were not statistically significant.

The factorial analysis of the carcass parameters are presented in Table 4.3.5. Neither type of soyabean meal nor protease addition significantly affected killing out % or the liver:carcass weight ratio. However, the lean:fat ratio was significantly increased ($P < 0.05$) by 0.99 (s.e.d. 0.13) and the pancreas:carcass weight ratio significantly decreased ($P < 0.001$) by 0.012 (s.e.d. 0.0027) (transformed data) in pigs fed MSB diets compared to RSB diets.

Table 4.3.3. Factorial analysis of adjusted means for performance data of pigs fed diets containing raw (R) or micronized (M) soyabean meal steeped for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units g^{-1} N of P3 with the live weight of pigs at the commencement of the trial as a covariate factor.

	Soyabean meal		Protease addition		
Parameter	Micronized	Raw	+	-	s.e.d.
Grower period					
ADFI (kg pig^{-1} d^{-1})	1.27	1.26	1.27	1.26	0.013
ADLWG (kg pig^{-1} d^{-1})	0.69^{x}	0.43^{x}	0.57	0.55	0.018
FCR	1.86^{x}	2.97^{x}	2.38	2.45	0.106
Finisher period					
ADFI (kg pig^{-1} d^{-1})	1.91^{x}	1.79^{x}	1.85	1.85	0.016
ADLWG (kg pig^{-1} d^{-1})	0.86^{x}	0.56^{x}	0.75^{a}	0.67^{a}	0.030
FCR	2.24^{x}	3.28^{x}	2.61^{a}	2.91^{a}	0.134
Overall					
ADFI (kg pig^{-1} d^{-1})	1.62^{x}	1.48^{x}	1.55	1.56	0.016
ADLWG (kg pig^{-1} d^{-1})	0.78^{x}	0.48^{x}	0.65^{a}	0.61^{a}	0.016
FCR	2.08^{x}	3.12^{x}	2.50^{a}	2.69^{a}	0.088

means with the same superscript for the same factor differ significantly ^{a} $P < 0.05$ ^{x} $P < 0.001$

Table 4.3.4. Analysis of interactions of adjusted means for performance data of pigs fed diets containing raw (R) or micronized (M) soyabean meal steeped for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units g^{-1} N of P3 with the live weight of pigs at the commencement of the trial as a covariate factor.

	Diet				
Parameter	M +	M -	R +	R -	s.e.d.
Grower period					
ADFI (kg pig^{-1} d^{-1})	1.28	1.26	1.25	1.26	0.019
ADLWG (kg pig^{-1} d^{-1})	0.70^{wx}	0.67^{yz}	0.43^{wy}	0.43^{xz}	0.025
FCR	1.82^{wx}	1.88^{yz}	2.93^{wy}	3.03^{xz}	0.121
Finisher period					
ADFI (kg pig^{-1} d^{-1})	1.92^{wx}	1.90^{yz}	1.79^{wy}	1.80^{xz}	0.016
ADLWG (kg pig^{-1} d^{-1})	0.89^{wx}	0.83^{yz}	0.61^{wya}	0.51^{xza}	0.041
FCR	2.172^{wx}	2.31^{yz}	3.04^{wya}	3.52^{xza}	0.190
Overall					
ADFI (kg pig^{-1} d^{-1})	1.63^{wx}	1.62^{yz}	1.48^{wy}	1.49^{xz}	0.014
ADLWG (kg pig^{-1} d^{-1})	0.80^{wx}	0.76^{yz}	0.50^{wy}	0.46^{xz}	0.023
FCR	2.016^{wx}	2.14^{yz}	2.99^{wy}	3.24^{xz}	0.125

means in the same row with the same superscript differ significantly ^{a} $P < 0.05$ ^{wxyz} $P < 0.001$

Table 4.3.5. Factorial analysis of adjusted means of carcass parameters of pigs fed raw or micronized soyabean meal steeped for 24 h at 20° C in the presence or absence of 20 000 units g^{-1} N of P3, with age at slaughter as a covariate factor.

	Factor 1: soyabean meal		Factor 2: protease pretreatment		
parameter	Raw	Micronized	+	-	s.e.d
killing out %	73	76	74	75	0.7
lean:fat*	1.85^{a}	2.86^{a}	2.47	2.25	0.122
liver:carcass weight	0.021	0.021	0.020	0.022	#
pancreas:carcass weight	0.0023^{a}	0.0018^{a}	0.0020	0.0021	#

supersripted means for the same factor in the same row are significantly different $^{a}(P < 0.05)$
* carcass weight and slaughter age were used as covariate factors for lean:fat
# untransformed data are presented for clarity. Significant differences were calculated from transformed data ($X' = 2 \arcsin \sqrt{X}$) s.e.d. of transformed data not presented

Table 4.3.6. Analysis of interactions for adjusted means of carcass parameters of pigs fed diets containing raw or micronized soyabean meal (factor 1) steeped for 24 h at 20° C in the presence or absence of 20 000 units g^{-1} N of P3. Age at slaughter was used as a covariate factor.

	Micronized		Raw		
parameter	+ P3	- P3	+ P3	- P3	s.e.d.
killing out %	75^{a}	77^{xd}	73^{d}	72^{xa}	0.1
lean:fat	2.91^{xy}	2.81^{wz}	2.02^{yw}	1.68^{xz}	0.183
liver:carcass weight	0.022	0.021	0.023	0.020	#
pancreas:carcass weight	0.0017^{fb}	0.0018^{ac}	0.0022^{bc}	0.0024^{fa}	#

superscripted means in the same row are significantly different $^{abc}(P < 0.05)$; $^{f}(P < 0.01)$; $^{xy}(P < 0.001)$
* carcass weight and slaughter age were used as covariate factors for lean:fat
# untransformed data are presented for clarity. Significant differences were calculated from transformed data ($X' = 2 \arcsin \sqrt{X}$) s.e.d. of transformed data not presented

There were no significant interaction effects on the carcass parameters between the type of soyabean meal and protease pretreatment of the diets. (Table 4.3.6)

The poor growth performance observed in the pigs fed RSB diets resulted in them being slaughtered at a lower mean slaughter weight of 73.6 kg (s.e.m. 1.02) compared with a mean slaughter weight of 91.8 kg (s.e.m. 1.02) for the pigs fed MSB diets. The pigs fed RSB diets were approximately 18 d (s.e.d. 0.07) older at slaughter than the pigs fed MSB diets. Because of the large difference in slaughter weight and age between the two groups, it was felt that the differences in these parameters may have confounded the outcome of the two factor factorial analysis. Therefore, the slaughter data were divided into two data sets according to the form of soyabean used and were analysed with protease pretreatment as the single factor. An additional parameter of the time taken to attain the minimum slaughter weight for that group was included in this analysis. These data are presented in Tables 4.3.7. and 4.3.8.

When the data were analysed in this manner the pretreatment of MSB with P3 significantly reduced ($P < 0.05$) the time taken for pigs to attain slaughter weight by approximately 5 days (s.e.m. 1.2). Pretreatment of RSB with P3 significantly increased lean : fat by 0.38 (s.e.m. 0.12) and liver : carcass weight by 0.029 (s.e.m. 0.007) (transformed data).

There was a considerable variation in the feed intake of pigs on the RSB diets particularly during the first week of the trial. The coefficients of variation (CV) for feed intake during the first week were 0.136, 0.064 and 0.156 for the total number of pigs, pigs fed MSB diets and pigs fed RSB diets respectively. By the second week this variability was reduced, CV's for total number of pigs, pigs fed MSB diets and pigs fed RSB diets were 0.093, 0.061 and 0.076 respectively.

Table 4.3.7. Analysis of adjusted means of carcass parameters for pigs fed micronized soyabean meal (M) steeped for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units g^{-1} N of P3

	Diet		
Parameter	M+	M-	s.e.d.
killout %[1]	76.1	76.6	0.59
lean : fat[2]	2.92	2.75	0.180
liver : carcass weight[1]	0.019	0.020	#
pancreas : carcass wt[1]	0.0019	0.0019	#
days to 90 kg[3]	75.9[a]	80.8[a]	1.72

[1] slaughter age as a covariate factor
[2] slaughter age and carcass weight used as covariate factors
[3] start weight used as a covariate factor
[a] means differ significantly ($P < 0.05$)
untransformed data are presented for clarity. Significant differences were calculated from transformed data ($X' = 2 \arcsin \sqrt{X}$) s.e.d. of transformed data not presented

Table 4.3.8. Analysis of adjusted means of carcass parameters for pigs fed raw soyabean meal (R) steeped for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units P3 g^{-1} N

	Diet		
Parameter	R+	R-	s.e.d.
killout %[1]	72.2	72.7	1.12
lean : fat[2]	2.07[a]	1.69[a]	0.121
liver : carcass weight[1]	0.025[a]	0.021[a]	#
pancreas : carcass wt[1]	0.0021	0.0022	#
days to 60 kg[3]	70.1	76.5	4.01

[1] slaughter age as a covariate factor
[2] slaughter age and carcass weight used as covariate factors
[3] start weight used as a covariate factor
[a] means differ significantly ($P < 0.05$)
untransformed data are presented for clarity. Significant differences were calculated from transformed data ($X' = 2 \arcsin \sqrt{X}$) s.e.d. of transformed data not presented

However, one of the pigs fed diet R + had consistently poor feed intake throughout the trial. The ranges of daily feed intake and daily weight gain over the grower and finisher periods are presented in Figure 4.3.1.a.-b. The distribution of overall daily feed intake and daily gain within each dietary group are in presented in Figure 4.3.2.a.-b.

As with Trial 1a there was some concern that the pigs with low feed intakes during the first 7 days of the trial would be compromised throughout the trial by being placed on a low point on the feeding scale initially. The mean growth curve and growth curves of individual pigs on each diet are presented in Figures 4.3.3 and 4.3.4.a.-d. Analysis of covariance (initial liveweight as the covariate factor) of the slopes of the growth curves is presented in Table 4.3.9. The growth rate of the pigs fed diets containing micronized soyabean was significantly ($P < 0.001$) increased by 0.31 (s.e.d. 0.02) kg liveweight gain d^{-1} compared with pigs fed diets containing raw soyabean. There was no significant effect on growth rate due to the pretreatment of either soyabean meal with 20 000 units g^{-1} N P3. Examination of the individual growth rates of pigs on each dietary treatment showed that there was a considerable degree of variation in growth rates among pigs fed diets containing raw soyabean, however the differences in growth rate were not statistically significant.

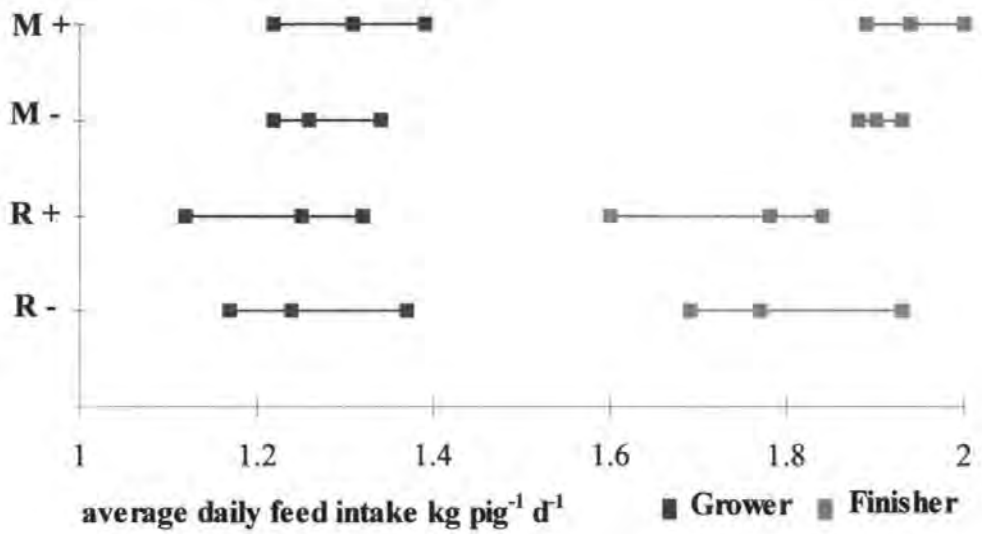


Figure 4.3.1a. Range of daily feed intake of pigs fed diets containing raw soyabean (R) or micronized soyabean (M) pretreated by steeping for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units g^{-1} N P3

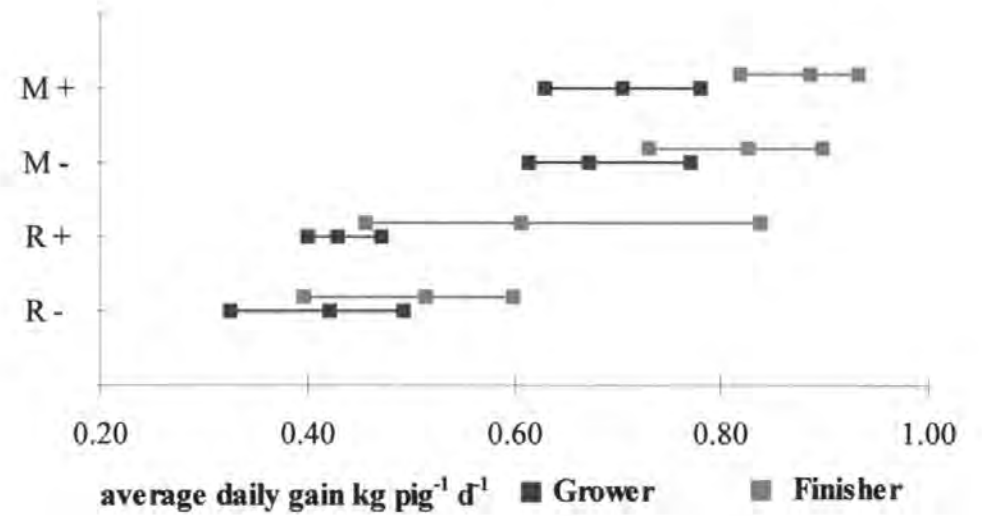


Figure 4.3.1.b. Range of daily weight gain of pigs fed diets containing raw soyabean (R) or micronized soyabean (M) pretreated by steeping for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units g^{-1} N P3

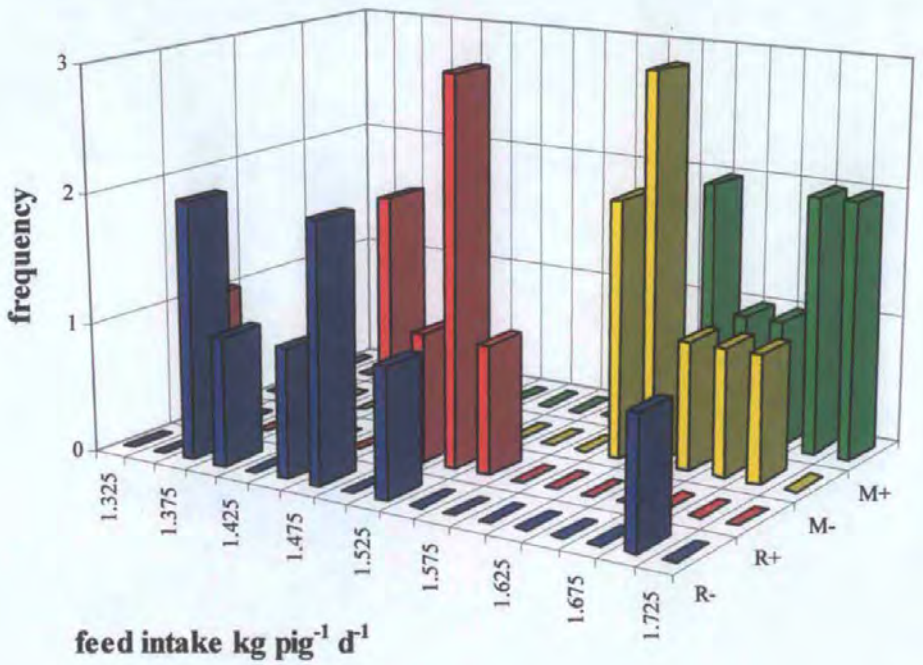


Figure 4.3.2.a Frequency histogram of overall average daily feed intake of pigs fed diets containing raw soyabean (R) or micronized soyabean (M) pretreated by steeping for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units g^{-1} N P3

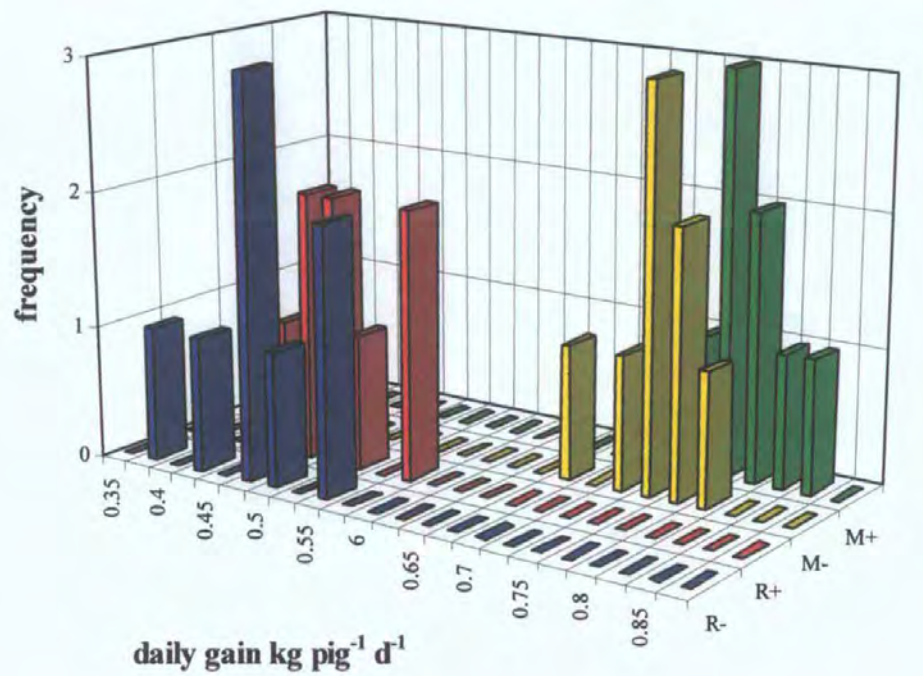


Figure 4.3.2.b Frequency histogram of overall average daily gain of pigs fed diets containing raw soyabean (R) or micronized soyabean (M) pretreated by steeping for 24 h at 20° C in the presence (+) or absence (-) of 20 000 units g^{-1} N P3

Table 4.3.9. Factorial analysis of the growth rate of pigs (kg weight gain d^{-1}) fed liquid diets containing raw or micronized soyabean meal steeped for 24 h at 20° C in the presence of absence of 20 000 units g^{-1} N P3

	Soya bean meal			
Protease P3 units g^{-1} N	Raw	Micronized	Main effect: protease	s.e.d.
0	0.46^{x}	0.77^{x}	0.61	0.02
20 000	0.48^{x}	0.80^{x}	0.64	
Main effect: soya	0.47^{x}	0.78^{x}		
s.e.d.	0.02			

^{x} means in the same row with the same superscript differ significantly ($P < 0.001$)
s.e.d for the interaction between soyabean meal and protease = 0.03

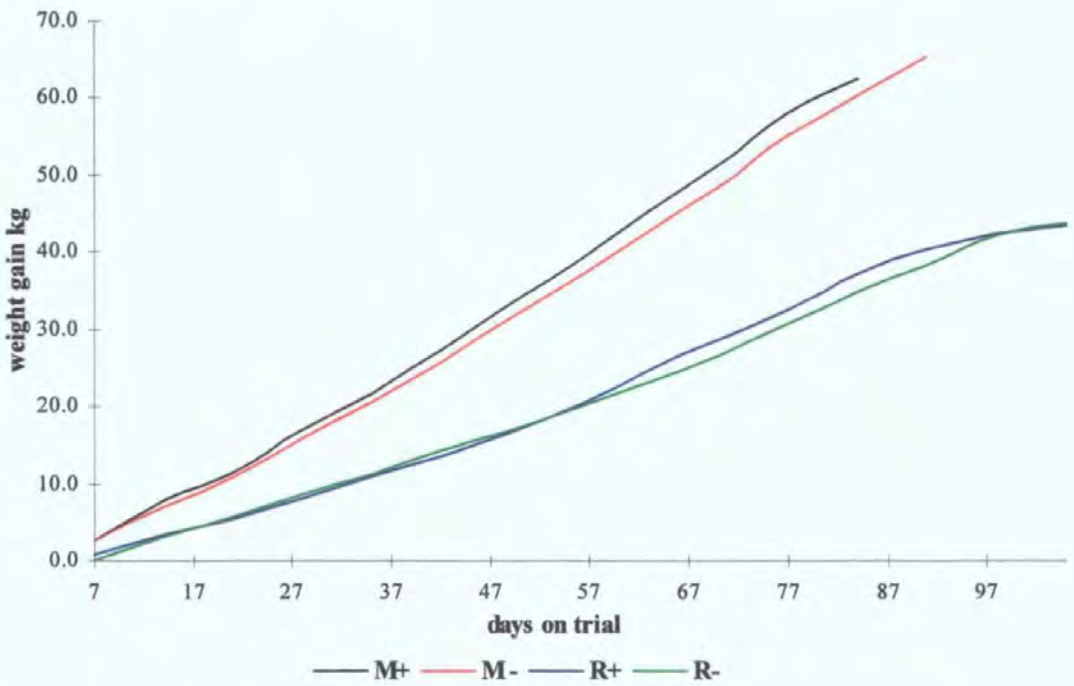
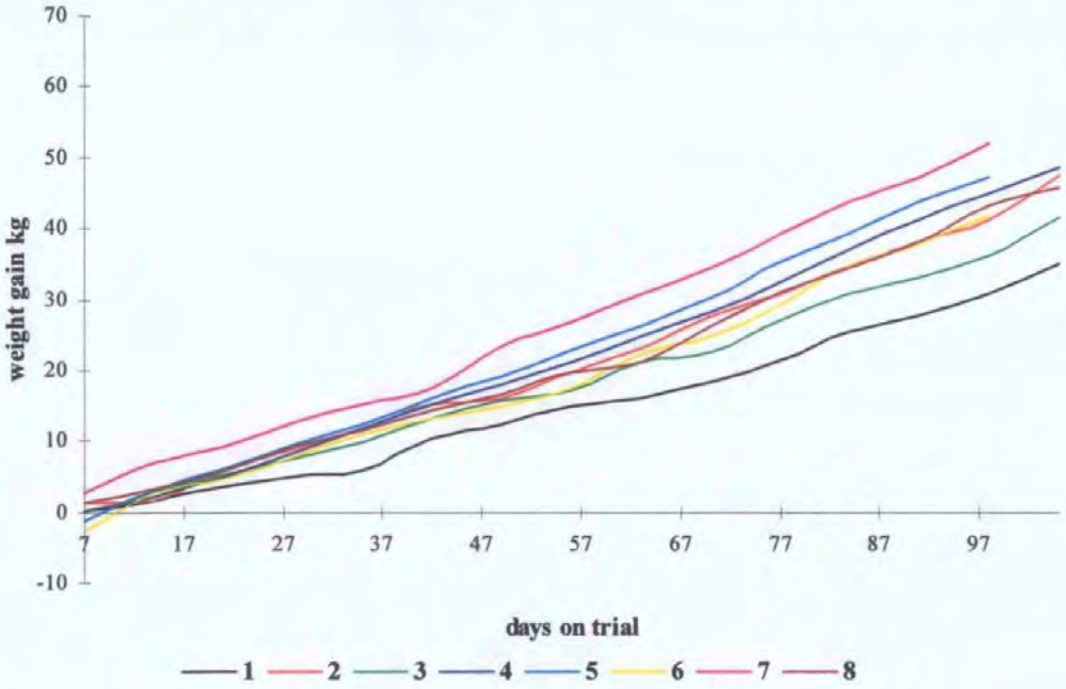
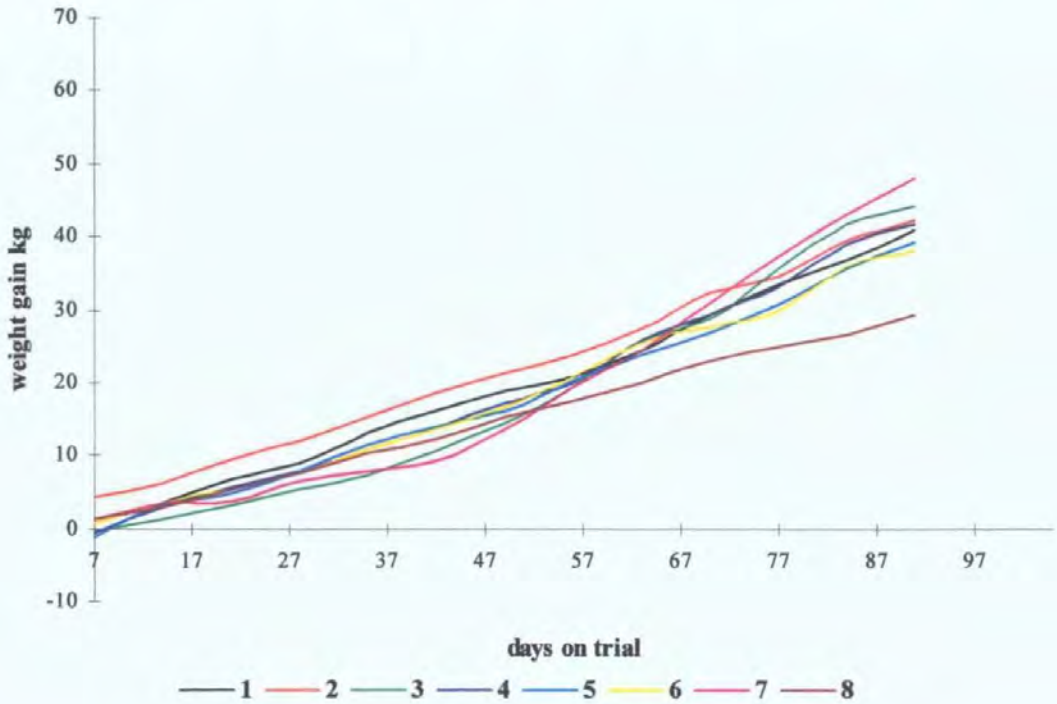


Figure 4.3.3. Mean (n = 8) growth rate of pigs (from day 7) fed diets containing raw (R) or micronized (M) soyabean pretreated with 0 (-) or 20 000 units g^{-1} N P3 (+). Trial 2.

Figure 4.3.4. Growth rates (from day 7) of individual pigs on each dietary treatment.

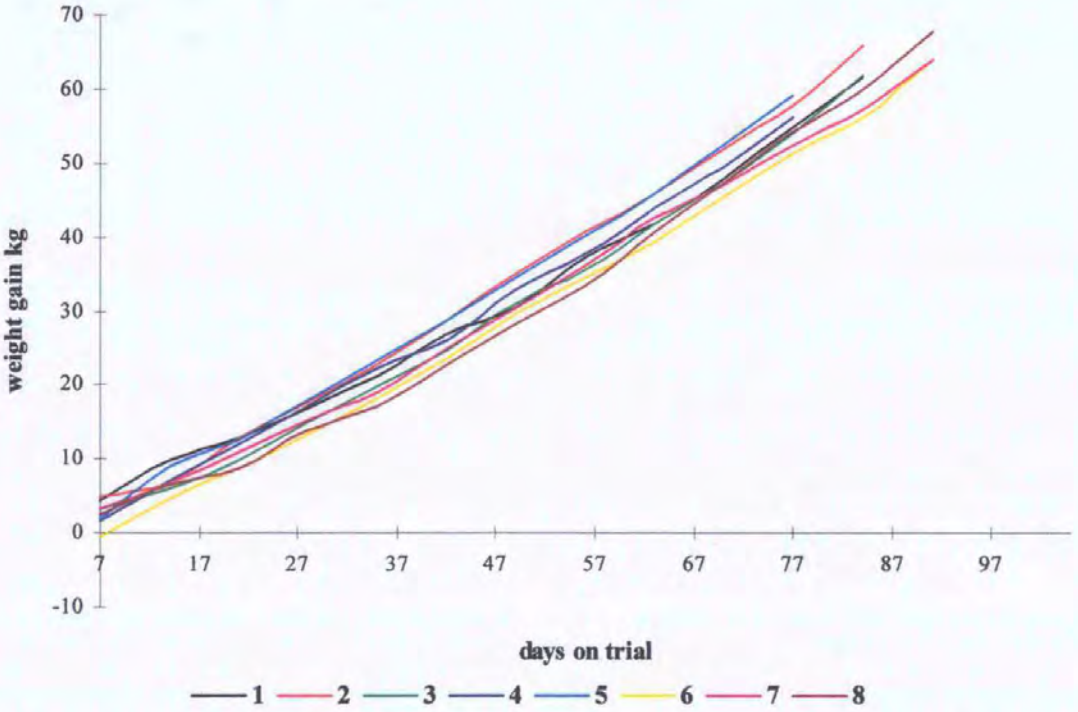


a) Growth rates of individual pigs fed diets containing raw soyabean meal steeped for 24 h at 20° C in the absence of P3 (R-)

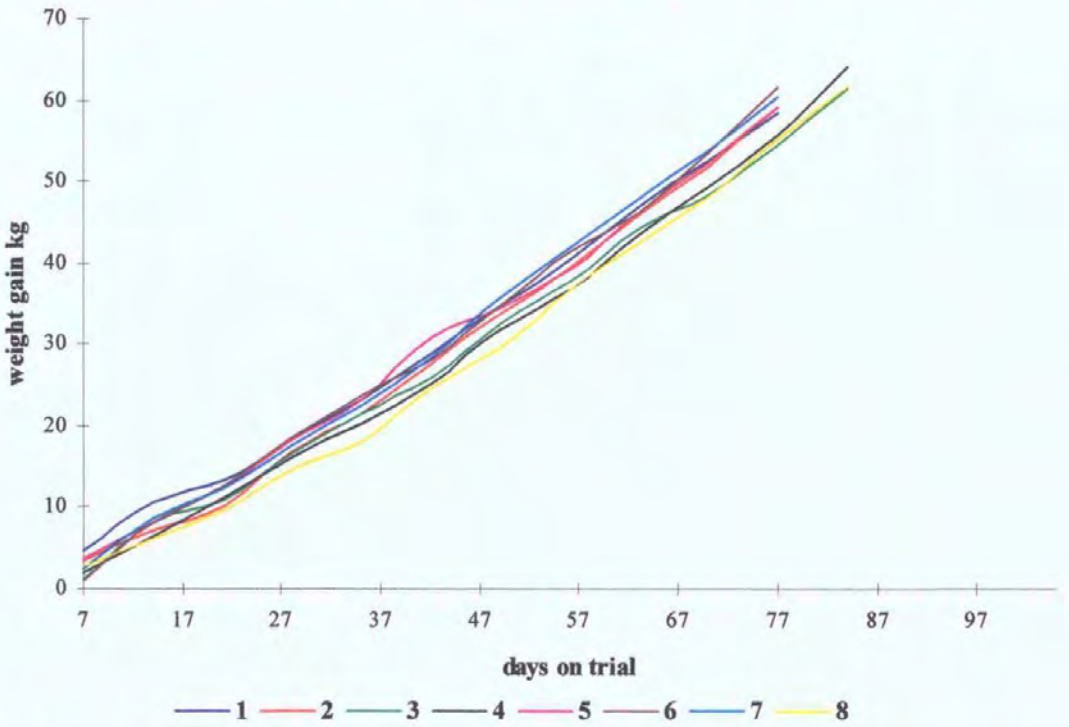


b) Growth rates of individual pigs fed diets containing raw soyabean meal steeped for 24 h at 20° C in the presence of 20 000 units g^{-1} N P3. (R+)

intercept on Y axis indicates weight gain / loss in the first 7 d



c) Growth rates of individual pigs fed diets containing micronized soyabean meal steeped for 24 h at 20° C in the absence of P3. (M-)



d) Growth rates of individual pigs fed diets containing micronized soyabean meal steeped for 24 h at 20° C in the presence of 20 000 units P3 g^{-1} N. (M+)

intercept on Y axis indicates weight gain / loss in the first 7 d

4.3.4 *Discussion.*

As with the previous Trial (1a) the main factor affecting the biological performance of the pigs was the form of soyabean in the diet. Crenshaw and Danielson (1985b) reported a 45 % reduction in performance (increase in FCR) for growing pigs (initial weight 23 kg) and a 30 % reduction in performance for finisher pigs (initial weight 68 kg) fed RSB diets compared with soyabean meal diets. They concluded that tolerance to RSB inclusion in the diet was age related. The results of this study tend to support their findings. The grower pigs suffered a 59 % reduction and the finisher pigs a 46 % reduction in performance due to RSB inclusion in the diet. However, analysis of the interaction between soyabean meal and P3 pretreatment revealed that P3 pretreatment increased the biological performance of the pigs fed RSB and to a lesser extent MSB. It is possible that the inclusion of the results for diets R+ and M+ in the factorial analysis were responsible for the differences in the reduction in performance between grower and finisher pigs fed RSB diets. Analysis of the data for diets M- and R - showed there was a reduction in growth performance of 59 % for the grower pigs and 51 % for the finisher pigs due to RSB consumption. So, although there does appear to be an age related tolerance to RSB it did not appear to be as great in this trial as that observed by Crenshaw and Danielson (1985b). However, in their study the two groups of pigs, 23 kg and 68 kg (initial weight) were fed RSB for 98 d and 28 d respectively. Therefore, the response they observed could have been due in part to the time pigs spent on RSB diets.

A greater range of feed intake was observed in pigs fed RSB diets (Figure 4.3.1a) which resulted in a greater range of growth rates (Figures 4.3.4a and 4.3.4b). Some of the pigs fed RSB diets had particularly poor growth rates, notably Pig 1 on diet R- (Figure 4.3.4a) and Pig 8 on diet R+ (Figure 4.3.4b). Although these were not statistically significant due to the overall variation in growth rates of pigs fed these diets they were considerably lower

than the other pigs on these diets. Pig 1 (R-) grew at a rate of 350 g live weight gain d^{-1} compared with 409 - 527 g live weight gain d^{-1} for the rest of the dietary group and Pig 8 (R+) grew at a rate of 357 g live weight gain d^{-1} compared with 460 - 573 g liveweight gain d^{-1} for the rest of the pigs on diet R+. These low growth rates were not apparently due to low feed allowance allocation as a consequence of poor initial feed intake, as the feeding records showed that both of these pigs frequently failed to consume all of their feed allowances throughout the trial. Two of the pigs fed diets R+ (Pig 3 and Pig 7, Figure 4.3.4b) had initial low growth rates which increased in the latter part of the trial. Interestingly the increase in growth rate coincided with the changeover from the grower to finisher diet. It was also noticed that most of the pigs fed raw soya diets appeared to consume their feed allowances more readily when they were fed the finisher diet. Although no explanation can be offered for this and bearing in mind that it was merely an observation, it could be speculated that this was due to the lower proportion of raw soya in the finisher diet.

The factorial analysis of carcass data showed a significant increase in the ratio of pancreas weight to carcass weight in the pigs fed RSB diets. This indicated that there was some degree of pancreas enlargement in the pigs fed RSB diets. Huisman and Tolman (1992) question the use of using pancreas weight relative to carcass weight or body weight as an indicator of pancreatic hypertrophy and suggest that absolute pancreas weight may give a better indication as the latter is not affected by reduced body weight due to consumption of antinutritional factors. The mean pancreas weight of the pigs fed MSB diets was 135 g compared with 115 g (s.e.m. 4.2) for pigs fed RSB diets. This indicated that there was no pancreatic hypertrophy in pigs fed RSB diets and that the differences in pancreas : carcass weight ratios were due to reduced carcass weight rather than increased pancreas weight. This agrees with the findings of Yen *et al.* (1974) and Schulze *et al.* (1993b) who report no

evidence of pancreatic hypertrophy in pigs fed either RSB or isolated soya trypsin inhibitors respectively. Furthermore, the mean pancreas weight of pigs fed RSB diets was significantly lower ($P< 0.01$) than that of pigs fed MSB diets. Huisman and Tolman (1992) reviewed the effects of feeding poorly digestible protein sources with high levels of SPI to monogastric farm animals. They concluded that the stimulatory effect of SPIs on pancreatic enzyme secretion, observed in small animals (rats and chicks), did not occur in larger animals. Also, feeding high levels of poorly digestible protein with high levels of SPI resulted in reduced pancreatic enzyme secretion which in turn could result in reduced pancreas size. In support of this, Schulze *et al.* (1993b) observed a reduction in pancreatic enzyme activity (trypsin annd chymotrypsin) in pigs fed diets containing 7.2 g kg^{-1} isolated KTI. However, they observed no reduction in pancreas size. The reduction in pancreas weight observed in the present study may reflect the duration of RSB consumption (*circa* 100 d) compared to the study of Schulze *et al.* (10 d).

The carcass parameters were also analysed as two separate data sets because it was not certain whether the large differences in slaughter weight and age could be sufficiently accounted for by including them as covariate factors in the two factor factorial analysis. Slaughter age and weight were included as covariate factors as there were slight variations in these parameters within each data set. For the pigs fed MSB diets this analysis revealed that the non significant numerical increases in ADG and FCR observed throughout the trial resulted in a significant reduction of 5 days (s.e.d. 1.7) in the time taken to attain slaughter weight. There were no other significant effects due to P3 pretreatment of MSB. In the pigs fed RSB diets there was a significant increase ($P < 0.05$) in lean : fat ratio of 0.38 (s.e.d. 0.12) due to pretreatment with P3. This indicated that the increases in ADG and FCR observed in the finisher period for pigs fed RSB pretreated with P3 was due to an increased utilization of RSB protein. It is noteworthy that in the analysis of interactions

between the form of soya and protease pretreatment (Table 4.3.6) the values obtained for the adjusted means of lean : fat in the pigs fed RSB diets differed by 0.34. However, the s.e.d in this case was 0.17, due to the larger error incurred within the data set, which resulted in the difference being non significant. There was also a significant ($P < 0.05$) increase in mean liver : carcass weight ratio of 0.029 (s.e.d. 009) (transformed data) in the pigs fed P3 treated RSB diets compared to untreated RSB diets. However, if Huisman and Tolman's (1989) suggestion, that absolute pancreas weight gives a better indication of hypertrophy than relative weight, holds true for all organ weights then the difference was not significant. Absolute liver weights were 1.18 and 1.30 kg (s.e.m. 0.06) for pigs fed diets R - and R + respectively ($P = 0.159$). Although the mean time taken to attain the minimum slaughter weight for the group was reduced from 76 to 70 days (s.e.m. 2.8) for the pigs fed raw soya pretreated with P3 this was not statistically significant. There was no significant difference in either pancreas:carcass weight ratio or absolute pancreas weight between pigs fed R + and R-. Mean absolute pancreas weights were 115 g for pigs fed R- compared with 114 g pigs fed R+ (s.e.m. 0.006). If the difference in pancreas weights between the pigs fed MSB diets and those fed RSB diets is a true reflection of a reduction in pancreas size due to long term consumption of increased levels of serine protease inhibitors, then the lack of a difference in pancreas weight between pigs fed R- and R+ would suggest that pretreatment with P3 had no biologically significant effect on the levels of serine protease inhibitors in the RSB.

4.4 Discussion and conclusions.

The pretreatment of RSB with exogenous proteases did not result in RSB attaining a similar nutritional value as processed soyabean. In both of the feeding trials (1a and 2) the pigs' growth responses to pretreatment of soyabean meals with exogenous proteases was greater for RSB than for ASB or MSB but in neither case did growth performance of pigs fed protease-pretreated RSB reach that of the pigs fed ASB or MSB. It was considered that it was unlikely that the response to protease pretreatment of RSB was due to any biologically significant reduction in trypsin inhibitor levels.

One of the factors that could have contributed to the greater response in the pigs fed RSB diets compared to pigs fed ASB or MSB diets was the dietary lysine:energy ratios to which the diets were formulated. The lysine:energy requirement of the pig decreases continuously with increasing liveweight (English *et al.* 1996; Gill, 1998). In formulating diets to cover a growth period in which liveweight is expected to increase by 30 - 40 kg a compromise situation is achieved. Pigs in the lower weight ranges may not be consuming sufficient protein to perform optimally whilst pigs in the higher weight ranges may be deaminating protein surplus to their requirements. Fowler (1984) demonstrated lysine:DE requirements of 0.79 g lysine MJ DE^{-1} for 30 kg pigs, 0.70 g lysine MJ DE^{-1} for 50 kg pigs and 0.59 g lysine MJ DE^{-1} for 90 kg pigs, whereas Gill (1998) reported requirements of 0.94, 0.80 and 0.66 g lysine MJ DE^{-1} for 30 kg, 50 kg and 90 kg pigs respectively. In both of the feed trials reported here lysine:DE ratios were reduced by ~ 12 % to enable any increase in protein digestibility due to protease treatment to be reflected in increased growth performance in the pig. In trial 1a the diets were formulated to provide 0.71 g lysine MJ DE^{-1} which would meet the requirements of a 50 kg pig (Fowler, 1984) or a 70 kg pig (Gill, 1998). In trial 1a some of the faster growing pigs exceeded 50 kg liveweight by the third week of the trial and 70 kg liveweight by the 5th week of the trial. Therefore,

for the faster growing pigs the ratio of lysine:DE may have been adequate in the latter part of the trial and they were unable to respond to any increase in protein digestibility due to protease pretreatment of the diet. All of the pigs with higher growth rates were fed ASB diets and this may partly explain the lack of a significant response to protease pretreatment of ASB.

A similar argument holds for Trial 2. The grower diet was formulated to contain 0.71 g lysine MJ DE^{-1} and the finisher diet to contain 0.63 g lysine MJ DE^{-1}. In this case all of the pigs were changed over to the finisher diet when their liveweight reached 50 kg. In both cases the lysine:DE ratio provided in the diets may have been adequate over some of the liveweight range for each group. This was particularly so for the finisher pigs fed MSB diets, as a diet containing 0.63 g lysine MJ DE^{-1} would probably be adequate for pigs approaching 90 kg liveweight. Therefore, the pigs would have shown little if any response to any increase in protein digestibility due to protease pretreatment of the diet as they attained heavier liveweights. For the pigs fed RSB diets in the finisher stage the situation was different because few of them attained liveweights of greater than 75 kg.

For the pigs fed RSB diets there was a significant response to pretreatment of RSB with proteases in the latter part of Trial 1a and in the finisher phase of Trial 2. The possible increase in tolerance to RSB with age has already been discussed. If this was the case it was possible that once the pigs had developed a tolerance to the antinutritional factors in RSB they were able to respond to any increase in protein digestibility due to protease pretreatment of RSB. Although the *in vitro* digestibility of nitrogen of RSB was significantly greater than for either of the SBM's used in these trials the *in vivo* digestibility was obviously less. The apparent ileal digestibility of lysine for RSB and heated soyabean meal have been reported as 57 % and 77 % (Yin *et al.* 1993) and 57.5 % and 74.4 %

(Herkelman *et al.* 1992) respectively. This means that the pigs fed RSB diets would have been more compromised with respect to lysine:DE in the diet than pigs fed MSB and therefore better able to respond to any increase in protein digestibility due to protease pretreatment.

In retrospect it was possible that the response to protease pretreatment of SBM's would have reached significance had dietary lysine:DE ratios been adjusted more frequently during the course of the trial.

In all of the studies that have been conducted to compare the growth response of pigs to the inclusion of RSB and full fat heat treated soyabean in the diet (Jiminez *et al.* 1963; Hanke *et al.* 1972; Herkelman *et al.* 1992; Friesen *et al.* 1993) it was assumed that the digestible energy of raw soya and heat treated soyabean was the same. The diets for these studies were formulated accordingly with the only difference between diets being the substitution of raw soya with an equal quantity of heat treated soya. In accordance with these studies the diets for the present trials were formulated assuming that the DE content of raw and autoclaved or micronized soya were identical. In other studies the authors have compared full fat raw soya to solvent extracted soyabean meal (Combs *et al.* 1967; Young 1967; Yen *et al.* 1974; Yen *et al.* 1977; Crenshaw and Danielson 1985b; Cook *et al.* 1988), and have adjusted the levels of soyabean meal in the diets to provide the same level of crude protein. There is little data available on the DE content of full fat raw soya. Published values for the DE content of full fat soyabean meals vary from 19.3 MJ kg^{-1} (Ewing 1997) to 15.59 MJ kg^{-1} (English *et al.* 1996) with intermediate values of 17.3 and 17.0 MJ kg^{-1} quoted by NRC (1998) and Whittemore (1993) respectively. In the diet formulations used in the present study the DE content of both raw and autoclaved or micronized soyabean meals were specified according to NRC guidelines at 16.9 MJ kg^{-1} (NRC 1988). However, Yin *et*

al. (1993) reported apparent ileal digestibility of gross energy of 18.07 MJ kg^{-1} for raw soyabeans compared to 19.20 MJ kg^{-1} for heated soyabeans. If the values of Yin *et al.* (1993) are substituted into the diet formulations for these studies this represents an increase of 0.35 and 0.69 MJ DE kg^{-1} for RSB and ASB diets respectively in Trial 1 which translates into lysine : energy ratios of 0.70 and 0.68 g MJ DE^{-1} respectively. For Trial 2 DE would have been increased by 0.38 and 0.31 MJ kg^{-1} for RSB diets and 0.77 and 0.69 MJ kg^{-1} for MSB diets in the grower and finisher diets respectively, giving respective values for lysine:energy ratios of 0.69 and 0.62 g MJ DE^{-1} for RSB diets and 0.67 and 0.60 g MJ DE^{-1} for MSB diets. These values are all slightly lower than the intended lysine:energy ratios. However, even the lowest values would still have approached the lysine:energy requirements over part of the weight range of the pigs on each diet. Therefore, it was likely that the raw soya diets and processed soya diets had slightly different lysine:energy ratios. However, this was unlikely to have a significant effect on the outcome of either trial because the small differences involved would have been outweighed by the overall effect of the inclusion of RSB in the diet.

In both Trial 1a and Trial 2 some of the pigs on the RSB diets had very low feed intakes during the first 7 d which resulted in low weight gain or weight loss during the first week of each trial. Consequently, they were allocated lower feed allowances according to the feeding scales used. However, this did not appear to result in reduced growth rates compared to the rest of the pigs in each dietary group as there were no significant differences in the regression coefficients within dietary groups. The pigs that suffered from low feed intake and weight loss initially had similar growth rates to those pigs that had higher initial feed intakes and weight gains within dietary treatments. Therefore, the reduced growth rate of pigs fed diets containing raw soya compared to pigs fed diets containing heat processed soya reflect the poor nutritional status of raw soyabean.

There appeared to be some benefit from pretreating raw soyabean with protease P3 or P4 but in neither Trial 1a nor Trial 2 did the pretreatment of raw soya with either protease raise its nutritional status to that of autoclaved soya or micrionized soyabean meal. Whether this was due entirely to the limited capability of these proteases to hydrolyse antinutritional factors such as serine protease inhibitors cannot be determined from these studies. Both P3 and P4 had small beneficial effects on ASB and MSB, although in both trials these were not statistically significant.

Chapter 5

Concluding Discussion.

Effects of proteases on target substrates.

The original aim of this programme of study was to determine whether the treatment of raw soyabean meal by steeping in the presence of proteases prior to inclusion in liquid feed systems for pigs could improve its nutritional value, thus making it economically and nutritionally viable to include in liquid diets for pigs.

Liquid feed systems lend themselves to the use of exogenous enzymes because individual 'problem' raw materials can be identified and subjected to enzyme treatment in an aqueous environment with no interference from other dietary ingredients. By pretreating the target raw material, in this case soyabean meal, the substrate concentrations of exogenous enzymes can be increased compared with treating a whole diet. It could be argued that in the case of raw soya the target substrate was the serine protease inhibitors. In this respect the pretreatments used in this study were not wholly successful because, although trypsin inhibitor levels were reduced by the action of the proteases, the reduction was not sufficient to bring trypsin inhibitor activity down to safe levels. Treatment with P2, P3 or P4 reduced trypsin inhibitor activity by 22 %, 37 % and 33 % respectively, whereas heat treatments used in commercially processed soyabean meals reduce trypsin inhibitor activity by ~ 90 % (Wright 1981). The lack of enzymatic degradation of serine protease inhibitors was not suprising as they represent only a small proportion (*circa* 6%) of the total protein (Nielsen 1983) and have been shown to retain their inhibitory activity even after cleavage of peptides from either side of the active site (Vaintraub and Haram 1995). However, if the target substrate for the proteases is taken to be soyabean protein, then some degree of success was attained. All proteases were active against the large molecular weight

components of glycinin and β-conglycinin and produced a partially hydrolysed soya product. This is in accordance with the work of Hessing *et al.* (1996) and Rooke *et al.* (1998) who reported partial hydrolysis of soyabean protein after pretreatment with exogenous proteases. The proteases they used (coded P1 and P2) were supplied by Finnfeeds International and information from the suppliers indicated that protease P1 used in their studies was the same enzyme as protease P3 used in the present studies. A significant degree of hydrolysis of glycinin and β-conglycinin in raw soyabean was observed after treatment with this enzyme (Hessing *et al.* 1996). Rooke *et al.* (1998) reported that different banding patterns in SDS PAGE gels were obtained with the proteases used in their study. In the studies reported here, the soyabean hydrolysates produced by treatment with P2, P3 or P4 also showed different banding patterns on SDS PAGE gels. This indicated that the proteases had different scissile bond specificities which resulted in them cleaving glycinin and β-conglycinin at different sites. However, as there was no information available on the bond specificities of the proteases it was impossible to confirm this.

The pretreatment of raw soyabean meal and a number of processed full fat soyabean meals with exogenous proteases resulted in an increased susceptibility to degradation by the digestive enzymes of the pig *in vitro*. Protease treatment resulted in increases in the *in vitro* digestibility of nitrogen of 6 - 8 % for raw soyabean (RSB), 4 - 7 % for steam pressure cooked soyabean (SPC), 3 - 8 % for micronized soyabean (MSB), 5 - 7 % for toasted soyabean (TSD) and 8 - 12 % for autoclaved soyabean (ASB). Although it has been suggested (Nielsen *et al.* 1988) that native soyabean storage protein is resistant to mammalian digestive enzymes the results of the study on the *in vitro* digestibility of nitrogen of raw and processed soyabean meals, reported in chapter 3, do not support this. These results showed the *in vitro* digestibility of nitrogen to be 76 % for RSB compared

with 67 - 80 % for the range of processed full fat soyabean meals. Therefore, the poor nutritional value of raw soyabean does not appear to be due to any inherent indigestibility of the protein. The findings of Hagemeister and Barth (1993) support this. They reported the disappearance of homoarginine in test meals containing guanidated raw soyabean meal and processed soyabean meal to be similar, 92 % and 96 % respectively. This led them to conclude that the poor growth response of pigs to the inclusion of raw soya in the diet is likely to be due to increased endogenous nitrogen losses.

The effect of pretreatment of raw soyabean and processed soyabean meals with exogenous proteases on growth performance in the pig.

It is generally assumed that the increased endogenous N losses incurred when pigs are fed raw soyabean are due to the presence of heat labile proteinaceous antinutritional factors, notably the serine protease inhibitors. Soyabean lectins (Liener 1994) and antigenic proteins (Stokes *et al.* 1987; Li, Nelssen, Reddy, Blecha, Klemm and Goodband 1991; Lalles *et al.* 1993; Dreau *et al.* 1993) have also been implicated in causing a poor growth response to the inclusion of raw soya in the diets of young pigs. The relative contributions of the latter two factors in eliciting a poor growth response in grower and finisher pigs is unclear. The antinutritional effects of soyabean lectins have been demonstrated in rats (Liener 1994) and the results have been extrapolated to other species. However, Jaffe (1980) concludes that there is no clear indication for any toxic effects of soyabean lectins. In addition the N-acetyl-D-galactosamine residues to which soyabean lectins bind have been demonstrated only on the enterocytes of young pigs (Classen *et al.* 1993). The presence of so called antigenic protein in raw soyabean should have little relevance in the diets of older pigs, because continous exposure to soya 'antigen' in the diet since weaning should have invoked oral tolerance by the time the pigs reach the grower stage.

However, it is clear from the work of Yen *et al.* (1974), Cook *et al.* (1988), Herkelman *et al.* (1992) and Li *et al.* (1998), all of whom compared the responses of pigs to diets containing raw soyabean of low or high trypsin inhibitor content, that serine protease inhibitors are not the only causative factor of poor growth in response to the inclusion of raw soyabean in the diet of pigs. Although lectins and 'antigenic' proteins may play some role in reducing growth, it remains unclear what the other antinutritive factors in raw soyabean are. Therefore, it may be supposed that if the pretreatment of raw soyabean with exogenous proteases was to have any effect on the growth performance of pigs it would be through the reduction of proteinaceous antinutritional factors.

None of the proteases used in these studies efficiently targeted the serine proteases in raw soyabean. Trypsin activity levels were reduced from 27.6 mg trypsin inhibited g^{-1} soyabean dry matter to 17.2, 18.5 and 20.0 mg trypsin inhibited g^{-1} soyabean DM after treatment with P3, P4 or P2 respectively (Table 2.7.3). Schulze *et al.* (1993a) suggest a threshold trypsin activity level of 2.49 mg trypsin inhibited g^{-1} diet, below which exogenous N losses do not significantly increase. The trypsin inhibitor activities of RSB treated with P4 in trial 1a would have provided 5.5 mg trypsin inhibited g^{-1} diet compared to 8.3 mg trypsin inhibited g^{-1} diet for untreated RSB. Those of the grower and finisher diets containing RSB treated with P3 in Trial 2 would have provided 5.5 and 5.0 mg trypsin inhibited g^{-1} diet respectively compared to 9.5 and 7.8 mg trypsin inhibited g^{-1} diet for the appropriate untreated RSB control diets. This reduction in trypsin inhibitor activity could explain the small but significant increases in growth response of the pigs fed diets containing protease treated raw soya in both trials. According to Schulze *et al.* (1993a) there is likely to be a dose response relationship between levels of SPI in the diet and exogenous and endogenous N losses. Therefore the pigs fed protease treated raw soya would have incurred a lesser degree of malabsorption of dietary N and loss of endogenous N than the pigs fed

raw soya that had received no protease treatment. The residual trypsin inhibitor activity present in the diets containing protease treated raw soya in both trials could explain the failure of the protease treatment to raise the nutritional value of raw soyabean to that of cooked soyabean.

The inclusion of RSB in the diet reduced feed intake which undoubtedly contributed to the poor performance in the pigs fed diets containing RSB. However, the poor response of pigs fed raw soyabean was unlikely to be entirely attributable to poor feed intake. The feeding scale for trial 2 was calculated from the voluntary feed intake of pigs fed diets containing RSB in trial 1b. Therefore, feed intake of all pigs reflected that of voluntary feed intake of RSB. However, feed conversion ratios of pigs fed RSB were greater by a factor of ~ 1.0 than those of pigs fed MSB. Herkelman *et al.* (1992) observed similar differences in FCR of pigs fed diets containing RSB and SBM when feed intake of all pigs was restricted to that of pigs fed diets containing RSB. This indicates that the reduction in growth of pigs fed RSB is a true reflection of the lower digestibility of RSB rather than a feed intake effect. The immediate effect on feed intake observed in the studies reported here, namely that the pigs refused to eat when initially offered the RSB diets, suggests that the problem was at least in part caused by palatability of raw soya. A point of interest was that the addition of P4 (Trial 1a) significantly increased feed intake of diets containing RSB by 0.13 kg pig^{-1} d^{-1} whereas the addition of P3 (Trial 2) had no effect. There are two possible explanations for this. Either the soya protein hydrolysate produced by P4 contained peptides that had a pleasant flavour which partially masked the unpleasant flavour of raw soya or P4 hydrolysed the factor that imparted the unpleasant taste.

The trend towards increased performance of the pigs fed protease treated ASB and MSB compared with the untreated counterparts could be a true reflection of an increase in

digestibility due to protease treatment. Processed soyabean meals are generally assumed to be highly digestible and studies on the true digestibility of amino acids from processed soyabean meals indicate that the true digestibility of lysine is 86 % (NRC 1998). Therefore, any increase in true lysine digestibility due to protease treatment is likely to be relatively small. The results of the *in vitro* N digestibility studies suggest that protease treatment may increase the availability of N by increasing the degree of degradation of soya protein into free amino acids and small peptides by the pigs' digestive enzymes. Whilst it may be assumed that this would increase the amount of N absorbed across the intestinal epithelia this might not be the case, because small increases in amino acid availability may not necessarily result in an increase in amino acid uptake. The uptake of amino acids and di/tri peptides is dependant on a number of factors including the type of protein, (Rerat and Corring 1991), feeding frequency (Batterham 1985) and the kinetics of amino acid transport mechanisms (Bastienelli and Sauvant 1999; Moughan 1999).

Cost benefits of protease treatments

The failure of protease treatment to reduce SPI levels sufficiently does not necessarily suggest that the treatment of raw soyabean with exogenous proteases is without value. The work of Young (1967) and Walker *et al.* (1987) indicated that raw soyabean could be used to replace 25 % of the soyabean meal fraction of diets for grower / finisher pigs with no detrimental effect of performance. This represents approximate inclusion levels of 6 % and 4 % raw soyabean in grower and finisher diets respectively. Using the figure of Schulze *et al.* (1993a) of a maximum trypsin inhibitor content of 2.49 mg trypsin inhibited g^{-1} diet and assuming raw soyabean to have a trypsin inhibitor activity of 30 mg g^{-1}, the maximum inclusion rate of raw soya would be 8.3 % which equates to 27 % of the soya component of a grower diet containing 30 % soyabean. By pretreating raw soya with P3 or P4 the trypsin inhibitor activity would be reduced and would allow the maximum inclusion levels

to increase to 13 % and 12 % respectively. This would represent 43 % of the soyabean fraction of a typical grower diet (SBM 30 %). In terms of costs the benefit of treating raw soya with these proteases is difficult to determine because the cost of production of the enzyme is unknown. However, some idea of the relative cost benefits can be determined. In Trial 2 the micronized soyabean cost approximately £24 tonne^{-1} more than the ground raw soyabean. If 27 % of the micronized soya were replaced with raw soyabean this would represent a saving of ~ £6 tonne^{-1} and if 43 % of the micronized soya were replaced with P3 treated raw soya this would represent a saving of ~ £10 tonne^{-1}. However, at the inclusion levels used in this trial, it took ~ 10 litres of P3 to treat a tonne of raw soyabean, which means the cost of the enzyme would have to be less than 40p per litre to make it cost effective.

In both feeding trials there were numerical increases in growth performance parameters due to the pretreatment of autoclaved or micronized soya with proteases. Although these increases were not statistically significant they were consistent throughout both trials and indicated that protease treatment did have some effect on both autoclaved and micronized soyabean meal.

In Trial 2 the pigs fed protease treated MSB performed slightly better than the pigs fed MSB that had received no protease treatment. Although, the increase in performance was not statistically significant it did result in the pigs fed protease-treated MSB attaining slaughter weight 5 days earlier on average than those fed untreated MSB diets. As a result of this the pigs fed MSB treated with P3 consumed 6.4 kg less feed per pig than those fed untreated MSB. The cost benefit of treating MSB with P3 in terms of increased performance leading to a reduction in time taken to reach slaughter weight can be calculated for this trial. The finisher diet cost £221 tonne^{-1}, therefore the pigs fed P3

treated MSB cost £1.41 less to produce than the pigs fed untreated MSB. Over the duration of the trial each pig consumed ~44.8 kg grower feed and ~84.2 kg finisher feed, with a total soya consumption of ~ 36 kg. It required 360 ml P3 to treat this quantity of MSB. Therefore, in order to be cost effective, the enzyme would have to cost less than £3.80 litre. This is a far more realistic figure than the cost benefit of treating raw soyabean.

Implications and future research.

As previously discussed (Chapter 4.5) the lysine:energy ratio at which the diets were formulated in the feeding trials may have had the effect of reducing the overall benefit of pretreating processed soyabean meals with proteases. The diets were formulated to cover the grower and finisher phases of pig production in accordance with current commercial practice. As Gill (1998) points out, considerable savings on feed costs may accrue if the diets continuously match protein and energy requirements. In effect this means increasing the number of diets fed during the production lifetime of the pig and this is only feasible where 'all in : all out' production is practised, and dietary changes can be matched to pens of pigs of approximately the same age and weight. Gill (1998) suggested three diets covering the growth periods from 30 - 50 kg, 50 - 70 kg and 70 kg to slaughter at either 88 kg or 110 kg. True phase feeding would require adjustment of dietary protein:energy ratios on a daily basis. Whilst the latter is obviously impractical when feeding dry diets, the use of computer assisted liquid feed systems in which different dietary components can be mixed on site immediately prior to feeding could enable diets to be tailored to meet the pigs' protein and energy requirements more efficiently. The use of proteases may be more valuable in such feeding regimes as any increase in protein availability due to protease pretreament could also be optimized. In the present studies the enzymes were probably working at sub-optimal temperatures and this required the used of relatively high inclusion levels of enzyme *circa* 0.5 % and 1% for P3 and P4 respectively in contrast to the 0.1 %

inclusion levels used by Caine *et al.* (1997b), Hessing *et al.* (1996) and Rooke *et al.* (1998). These authors used pretreatment temperatures of 50° C for shorter time periods, namely 3 h (Hessing *et al.* (1996) and Rooke *et al.* (1998)) or 16 h (Caine *et al.* (1997b)). If the benefits of enzyme pretreatment in terms of increases in pig production were great enough the energy costs of heating pretreatment tanks may be warranted. The enzymes currently produced for use in the animal feed industry have not been developed with pretreatment regimes in mind. Therefore most commercially available enzyme applications probably have relatively high temperature optima as the intended site of activity is the digestive tract of the animal. With pretreatment regimes it may be more feasible to develop enzyme preparations with low temperature optima in order to save energy costs of pretreatment.

Although the present studies indicate that the cost benefits of using proteases in pig diets may be small in terms of pig production, the possibility that they may be beneficial in reducing nitrogen pollution in pig effluent have not been explored. The presentation of partially hydrolysed protein, in which the availability of dietary amino acid is increased, in conjunction with phase feeding systems may reduce N excretion. This would require a value, in terms of increased amino acid availability and nitrogen utilization, to be placed on protease-treated protein supplements. Diets could then be formulated to minimize N excretion. The information needed to do this cannot be elucidated from the data obtained from the present studies. However, the ileal digestibility and nitrogen balance studies required represent an area for further research.

In terms of increasing protein digestibility the choice of substrates used in this project was probably not ideal. Raw soyabean is a 'problem' substrate in that the presence of antinutritional factors such as serine protease inhibitors have a large impact on its digestibility and therefore its suitability for use in pig diets. Treatment with the proteases

used in this study did not sufficiently reduce levels of ANF's and raise its nutritional status. On the other hand, the processed soyabean meals, ASB and MSB were not sufficiently indigestible for protease treatment to have any significant impact on their utilization by grower or finisher pigs. However, it is possible that protease treatment may have a greater impact on the protein digestibility of vegetable protein meals such as lupin or sunflower meal in which ANF's do not appear to be such a problem but digestibility is less than for soya.

In the present studies only proteases were used in the pretreatment of raw or processed soyabean meals. However, it is possible that synergistic combinations of proteases and non-starch polysaccharide degrading enzymes may be more effective, as hydrolysis of non-starch polysaccharides may facilitate the accessibility of proteins for degradation by proteases. Studies in which multi-enzyme complexes have been added to dry diets have shown some increases in performance due to enzyme addition. Spring *et al.* (1998) observed improvements in FCR of 8.8 % in piglets fed corn/soya diets supplemented with a mixture of protease, cellulase, pentosanase, α-galactosidase and amylase compared with piglets fed unsupplemented diets. Nasi (1991) observed a trend towards increased N retention in pigs fed diets supplemented with cellulase, protease and β-glucanase compared with pigs fed unsupplemented diets. Any synergistic effects between the enzymes in these studies were not investigated and it is likely that such multi-enzyme mixtures arise as crude fermentation extracts from which individual enzymes are not separated.

The above all represent areas for future research. Although there is a huge potential for the manipulation of dietary components through enzyme applications in liquid feed systems future research in this area is likely to be driven by commercial and economic concerns. The cost benefits of pretreatment of protein components of pig diets with exogenous

proteases in terms of pig production are likely to be small. However, the implementation of legislation concerning nitrogenous pollution from farm effluent and the implications this has for encouraging increased efficiency of nitrogen utilization in the pig may provide the impetus for more research in this area.

APPENDICES

Appendix 1

Protease assay method

One unit of protease activity is the amount of enzyme required to liberate 1 μg of phenolic compound (expressed as tyrosine equivalents) in 1 min under the conditions of the assay.

Method.

Serial dilutions of a 100μg ml^{-1} tyrosine (in 0.02 M NaCl) stock solution were prepared in order to construct a standard curve.

One ml of an appropriate dilution of P3 or P4 was mixed with 5 ml 0.6% (w/v) casein at pH 7.5 subsrtate and incubated at 40° C for 30 min. Five ml of precipitation reagent (0.115 M trichlotracetic acid, 0.22 M sodium actetate, 0.3 M acetic acid) was added and the mixture incubated for a further 30 min at 40° C and immediately filtered through filter paper (Whatman 1). Two ml of the filtrate was mixed with 5 ml 0.55 M Na_2CO_3 and 1ml Folin - Ciocalteau reagent and incubated at 40 ° C for 30 min. After cooling to room temperature the absorbance was measured at 660 nm against distilled water. Appropriate enzyme blanks were prepared by incubation with the precipitation reagent prior to the addition of casein. Three replicate analyses of each enzyme dilution were conducted.

The enzyme activity was calculated using the equation:-

$$\text{Activity} = \frac{(A_x - A_o)FD_f}{kt} \qquad \text{equation 1}$$

where A_x = absorbance of enzyme sample, A_o = absorbance of enzyme blank, k = slope of the tyrosine standard curve, F = reaction dilution factor, D_f = sample dilution factor, t = reaction time.

The above procedure was repeated for protease P2 with the pH of the casein substrate adjusted to pH 3.5.

Results.

The tyrosine standard curve is presented in Figure 1. The enzyme activity calculated for P2 was 424 181 units g^{-1} and for P3 and P4 enzyme activity was 59 583, and 247 866 units ml^{-1} respectively.

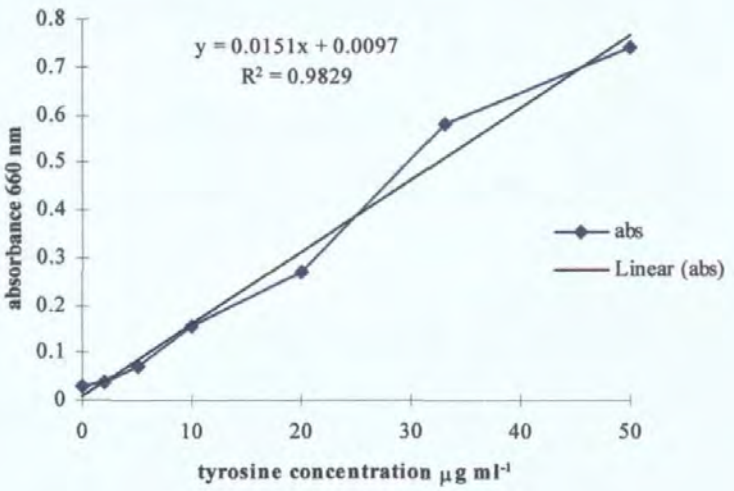


Figure 1 Tyrosine standard curve

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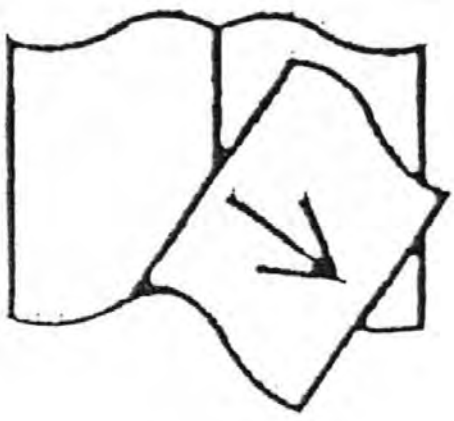


Appendix 2

Adaptation of the A.O.A.C. (1992) method for the determination of α-amino nitrogen in wort.

The A.O.A.C. (1992) method for the determination of α-AN was adapted to allow the heating and cooling stages to be undertaken in a thermocycler (PCR machine) as this allows accurate timing of the heating and cooling stages.

Method 1. Standard A.O.A.C. Method

For sample, standard and blank reagent tubes, 1.0 ml ninhydrin colour reagent (10 g $Na_2HPO_4.12\ H_2O$, 6 g KH_2PO_4, 0.5 g 1,2 3 -indantrione.H_2O, 0.3 g fructose in 100 ml distilled H_2O) was added to 2 ml of sample, 2 ml glycine standard (1.07 μg ml^{-1}) and 2 ml distilled water respectively in glass 16 x 150 mm test tubes. Reagent tubes were stoppered with glass marbles and heated in a boiling water bath at 100° C for exactly 16 min then transferred to a 20° C bath for 20 min. Five ml diluent (0.2 % KIO_3 in 40 % ethanol) was added to each tube, mixed thoroughtly and the absorbance at 570 nm measured after 20 min.

Method 2. Adapted Method.

For sample, standard and blank reagent tubes, 0.125 ml ninhydrin colour reagent was added to 0.25 ml sample, glycine standard or distilled watrer respectively in 0.5 ml Eppendorf tubes. The tubes were capped and placed in a thermocycler which was programmed for 16 min at 99° C followed by 20 min at 20° C. The contents of each reagent tube were removed and mixed with 0.625 ml diluent in a 1 ml cuvette. Absorbance at 570 nm was measured after 20 min.

The above methods were used to determine α-AN in a range of glycine standards and in a set of 12 samples of raw soya slurry.

α-AN in the slurries was calculated using the equation

$$\alpha\text{-AN} = \frac{(A_s - A_b)}{(A_{std} - A_b)} x0.002x\frac{D}{S} \qquad \text{equation 1}$$

where A_s, A_{std} and A_b = absorbance of sample, standard and blank respectively, D = dilution factor and S = g soya in sample. The standard contains 0.002 mg α-AN ml^{-1} .

Results

The standard curves obtained for a range of glycine standards with Method 1 (M1) and Method 2 (M2) are presented in Figure 1. The α-AN detected in the set of 12 raw soya samples is presented in Table 1.

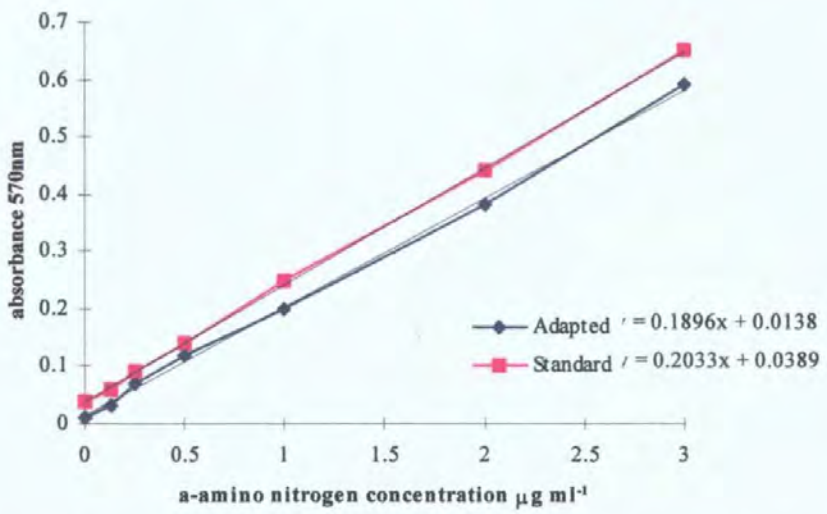


Figure 1. Standard curve for α amino nitrogen from glycine using A.O.A.C. method (M1) or adapted method (M2)

Table 1 α-AN (mg g^{-1} soya) detected in 12 samples of raw soyabean slurry with the A.O.A.C. standard method (M1) or the adapted method (M2).

	Method			CV %	
sample	M1	M2	s.e.d.	M1	M2
1	0.91	0.99	0.02[a]	3.7	0.2
2	0.98	1.06	0.02[a]	3.4	0.2
3	0.78	0.88	0.02[b]	4.3	0.2
4	1.31	1.39	0.04	3.9	0.4
5	1.24	1.40	0.03[b]	4.1	0.2
6	1.32	1.44	0.02[b]	2.5	0.1
7	1.52	1.66	0.02[b]	2.3	0.2
8	1.53	1.69	0.03[b]	3.4	0.2
9	1.55	1.70	0.02[b]	2.2	0.2
10	1.13	1.28	0.03[b]	4.5	0.4
11	1.09	1.19	0.02[b]	3.5	0.7
12	1.12	1.21	0.01[b]	1.7	0.9

[a] means are significantly different ($P < 0.05$)
[b] means are significantly different ($P < 0.01$)

The amount of α-AN detected by method 2 was significantly higher by 0.121 (s.e.d. 0.06) mg g^{-1} soya than that detected by method 1. The variability between the absorbance readings within each sample was considerably less with method 2 than with method 1.

Appendix 3

Method for predicting the total tract digestibility of energy in feedstuffs and pig diets by *in vitro* analyses. Boisen and Fernandez (1997)

Step 1

Approximately 0.5 g finely ground material (<1mm) were weighed to an accuracy of ± 0.1 mg in 100 ml conical flasks. In each of the series a blank was included. A small magnetic rod and 25 ml of phosphate buffer (0.1M, pH 6.0) were added to each flask and sample and buffer were mixed carefully by gentle magnetic stirring. To the mixture was added 10 ml 0.2 M HCl, and pH was adjusted to pH 2.0 with a 1M HCl or a 1M NaOH solution. To the mixture was then added 1 ml of freshly prepared pepsin solution containing 25 mg pepsin (porcine, 2000 FIP-U g^{-1} Merck No. 7190). In order to prevent bacterial growth, 0.5 ml of a Chloramphenicol solution (0.5 g Chloramphenicol, Sigma No. C-0378, per 100 ml ethanol) was added. Then the flasks were closed with a rubber stopper and placed on multipoint stirrers (Variomag multipoint HP 15) in a thermostatically controlled heating chamber (Salvis SA) at 39° C and the samples were stirred gently for two hours.

Step 2

To the mixture was added 10 ml of a phosphate buffer (0.2M, pH 6.8) + 5 ml of a 0.6 M NaOH solution. The pH was then adjusted to pH 6.8 with a 1 M HCl or 1 M NaOH solution. The slurry was then carefully mixed with 1 ml of a freshly prepared pancreatin solution containing 100 mg pancreatin (porcine, grade IV, Sigma No. P1750; undissolved tissue material was removed after centrifugation). After closing with a rubber stopper, the flasks were incubated under continuous magnetic stirring at 39° C for 4 h.

Step 3

To the mixture was added 10 ml of a 0.2 M EDTA solution. The pH was adjusted to pH 4.8 with 30 % acetic acid. The resulting slurry was mixed with 0.5 ml of a multi-enzyme complex containing a wide range of microbial carboanhydrases including arabinase, β-glucanase, hemicellulase, xylanase and pectinase (Viscozyme 120 L, 120 FBG g^{-1} , Novo-Nordisk, Bagsvaerd, Denmark). After closing with a rubber stopper, the flasks were incubated under continous magnetic stirring at 39° C for 18 h. The undigested residues were then collected in a filtration unit (Fibertec System M, Tecator , Sweden) by using dried and pre-weighed glass filter crucibles (d: 3cm; pore size: 40 - 90 μ) containing about o.4 g celite (545, Tecator, preheated at 500 ° C for 4 h) as a filter aid. All material was transferred with water to the crucible. After consecutive washings (3 min each time) with 2 x 10 ml ethanol (96 %) and acetone (99.5 %) respectively, the undigested residues were dried at 130° C until constant weight (for obtaining values of dry matter digestibility) and then ashed at 500° C for 4 h.

Appendix 4. **Plans of test facilities**

Figure 1. Plan of test facility used in Trial 1a

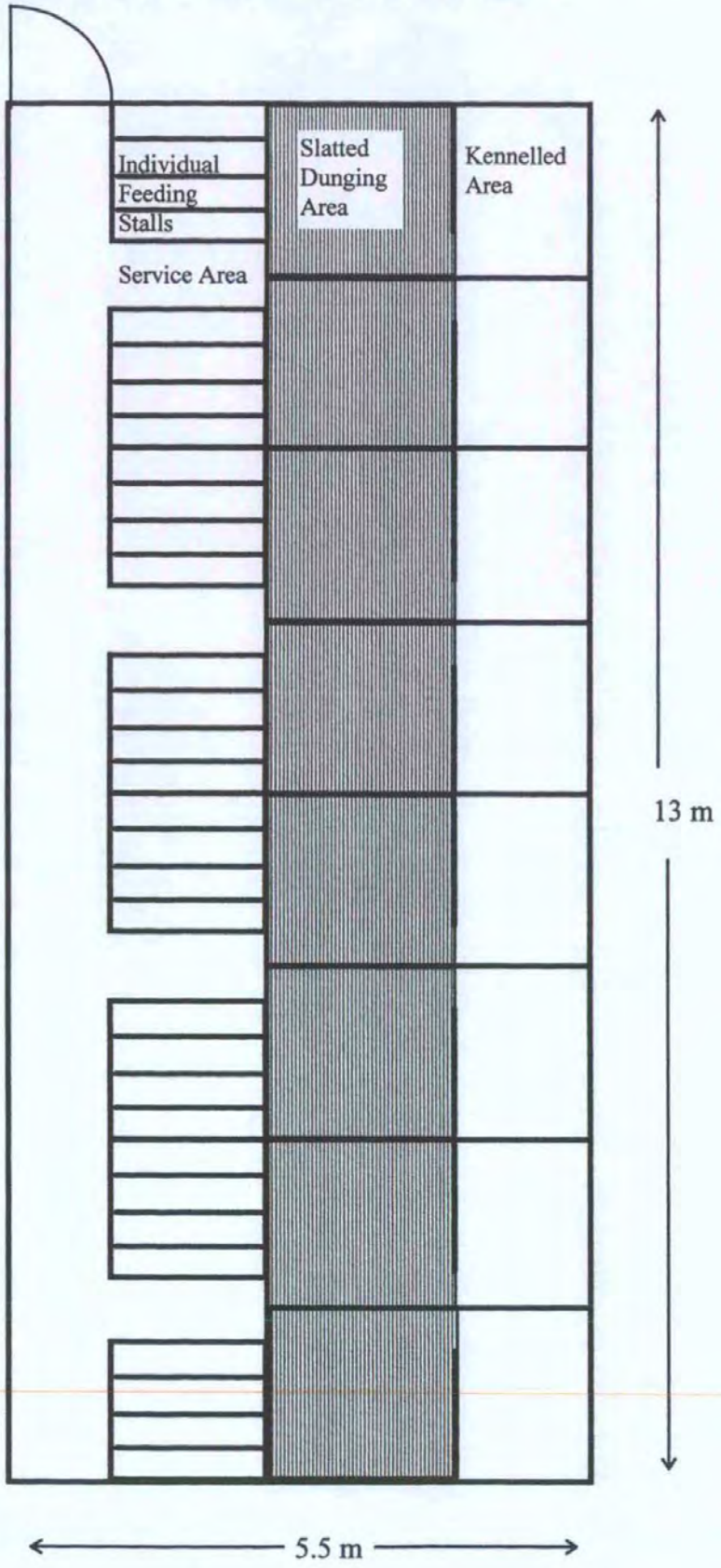
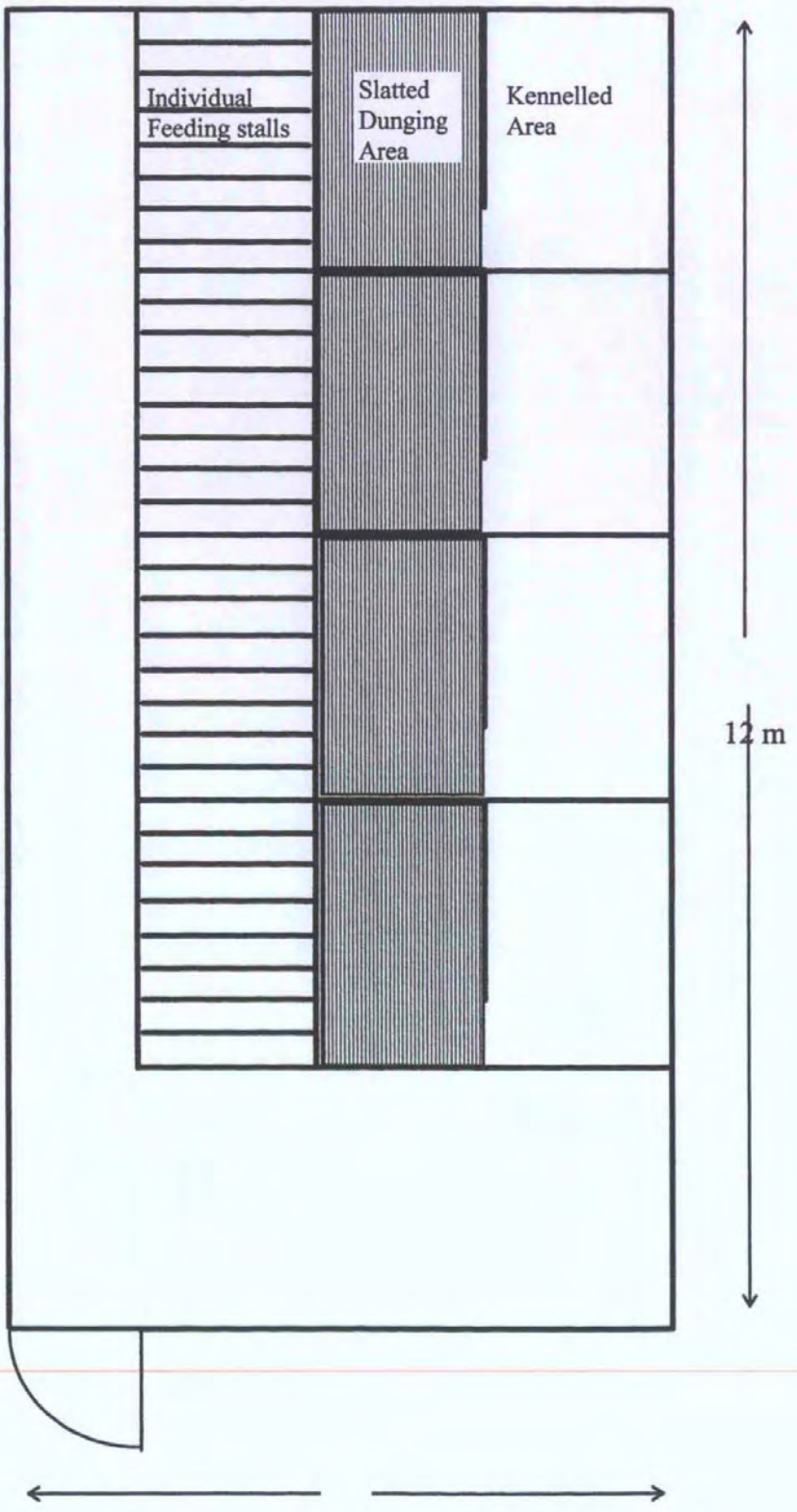


Figure 2. Plan of test facility used in Trial 1b and Trial 2.



Appendix 5 Feeding Scales

A5.1 Feeding scale used in Trial 1a.

liveweight	metabolic body wt $W^{.63}$	DE intake 2.4 $W^{0.63}$	90% feed intake (DE/14)	feed per meal (g)	soya (g)	enzyme (ml)	basal diet (g)
26	7.79	18.69	1.20	601	180	0.45	421
27	7.98	19.14	1.23	615	185	0.46	431
28	8.16	19.58	1.26	630	189	0.47	441
29	8.34	20.02	1.29	644	193	0.48	451
30	8.52	20.45	1.31	657	197	0.49	460
31	8.70	20.88	1.34	671	201	0.50	470
32	8.88	21.30	1.37	685	205	0.51	479
33	9.05	21.72	1.40	698	209	0.52	489
34	9.22	22.13	1.42	711	213	0.53	498
35	9.39	22.54	1.45	725	217	0.54	507
36	9.56	22.94	1.48	738	221	0.55	516
37	9.73	23.34	1.50	750	225	0.56	525
38	9.89	23.74	1.53	763	229	0.57	534
39	10.05	24.13	1.55	776	233	0.58	543
40	10.22	24.52	1.58	788	236	0.59	552
41	10.38	24.90	1.60	800	240	0.60	560
42	10.54	25.28	1.63	813	244	0.61	569
43	10.69	25.66	1.65	825	247	0.62	577
44	10.85	26.04	1.67	837	251	0.63	586
45	11.00	26.41	1.70	849	255	0.64	594
46	11.16	26.78	1.72	861	258	0.65	602
47	11.31	27.14	1.74	872	262	0.65	611
48	11.46	27.50	1.77	884	265	0.66	619
49	11.61	27.86	1.79	896	269	0.67	627
50	11.76	28.22	1.81	907	272	0.68	635
51	11.91	28.57	1.84	918	276	0.69	643
52	12.05	28.93	1.86	930	279	0.70	651
53	12.20	29.28	1.88	941	282	0.71	659
54	12.34	29.62	1.90	952	286	0.71	667
55	12.49	29.97	1.93	963	289	0.72	674
56	12.63	30.31	1.95	974	292	0.73	682
57	12.77	30.65	1.97	985	296	0.74	690
58	12.91	30.99	1.99	996	299	0.75	697
59	13.05	31.32	2.01	1007	302	0.76	705
60	13.19	31.66	2.03	1017	305	0.76	712
61	13.33	31.99	2.06	1028	308	0.77	720
62	13.47	32.32	2.08	1039	312	0.78	727
63	13.60	32.64	2.10	1049	315	0.79	734
64	13.74	32.97	2.12	1060	318	0.79	742
65	13.87	33.29	2.14	1070	321	0.80	749
66	14.01	33.61	2.16	1080	324	0.81	756
67	14.14	33.93	2.18	1091	327	0.82	764
68	14.27	34.25	2.20	1101	330	0.83	771
69	14.40	34.57	2.22	1111	333	0.83	778
70	14.53	34.88	2.24	1121	336	0.84	785
71	14.67	35.20	2.26	1131	339	0.85	792
72	14.80	35.51	2.28	1141	342	0.86	799
73	14.92	35.82	2.30	1151	345	0.86	806

A5.2. Feeding Scales used in Trial 2

Grower diet

liveweight (kg)	MBW $W^{0.63}$	ad lib DE intake $2.24W^{0.63}$	90% ad lib feed intake (DE/14)	feed/meal (g)	soya @31.84% (g)	basal diet (g)	enzyme (ml)
20	6.60	14.79	951	475	151	324	1.5
21	6.81	15.25	980	490	156	334	1.6
22	7.01	15.70	1009	505	161	344	1.6
23	7.21	16.15	1038	519	165	354	1.7
24	7.41	16.59	1066	533	170	363	1.7
25	7.60	17.02	1094	547	174	373	1.7
26	7.79	17.45	1121	561	179	382	1.8
27	7.98	17.87	1148	574	183	391	1.8
28	8.16	18.28	1175	588	187	400	1.9
29	8.34	18.69	1201	601	191	409	1.9
30	8.52	19.09	1227	614	195	418	2.0
31	8.70	19.49	1253	626	199	427	2.0
32	8.88	19.88	1278	639	203	436	2.0
33	9.05	20.27	1303	652	207	444	2.1
34	9.22	20.66	1328	664	211	453	2.1
35	9.39	21.04	1352	676	215	461	2.2
36	9.56	21.42	1377	688	219	469	2.2
37	9.73	21.79	1401	700	223	477	2.2
38	9.89	22.16	1424	712	227	485	2.3
39	10.05	22.52	1448	724	231	493	2.3
40	10.22	22.88	1471	736	234	501	2.3
41	10.38	23.24	1494	747	238	509	2.4
42	10.54	23.60	1517	759	242	517	2.4
43	10.69	23.95	1540	770	245	525	2.5
44	10.85	24.30	1562	781	249	532	2.5
45	11.00	24.65	1584	792	252	540	2.5
46	11.16	24.99	1607	803	256	548	2.6
47	11.31	25.33	1628	814	259	555	2.6
48	11.46	25.67	1650	825	263	562	2.6
49	11.61	26.01	1672	836	266	570	2.7
50	11.76	26.34	1693	847	270	577	2.7
51	11.91	26.67	1714	857	273	584	2.7
52	12.05	27.00	1736	868	276	591	2.8

Finisher diet

liveweight (kg)	MBW $W^{0.63}$	ad lib DE intake $2.24W^{0.63}$	90% feed int (DE/13.5)	feed /meal (g)	soya @26% (g)	basal diet (g)	enzyme (ml)
51	11.91	26.67	1778	889	231	658	2.3
52	12.05	27.00	1800	900	234	666	2.3
53	12.20	27.32	1822	911	237	674	2.4
54	12.34	27.65	1843	922	240	682	2.4
55	12.49	27.97	1865	932	242	690	2.4
56	12.63	28.29	1886	943	245	698	2.5
57	12.77	28.61	1907	954	248	706	2.5
58	12.91	28.92	1928	964	251	713	2.5
59	13.05	29.23	1949	974	253	721	2.5
60	13.19	29.54	1970	985	256	729	2.6
61	13.33	29.85	1990	995	259	736	2.6
62	13.47	30.16	2011	1005	261	744	2.6
63	13.60	30.47	2031	1016	264	752	2.6
64	13.74	30.77	2051	1026	267	759	2.7
65	13.87	31.07	2072	1036	269	766	2.7
66	14.01	31.37	2092	1046	272	774	2.7
67	14.14	31.67	2111	1056	274	781	2.7
68	14.27	31.97	2131	1066	277	789	2.8
69	14.40	32.26	2151	1075	280	796	2.8
70	14.53	32.56	2171	1085	282	803	2.8
71	14.67	32.85	2190	1095	285	810	2.8
72	14.80	33.14	2209	1105	287	817	2.9
73	14.92	33.43	2229	1114	290	825	2.9
74	15.05	33.72	2248	1124	292	832	2.9
75	15.18	34.00	2267	1133	295	839	2.9
76	15.31	34.29	2286	1143	297	846	3.0
77	15.43	34.57	2305	1152	300	853	3.0
78	15.56	34.86	2324	1162	302	860	3.0
79	15.69	35.14	2342	1171	305	867	3.0
80	15.81	35.42	2361	1181	307	874	3.1
81	15.93	35.69	2380	1190	309	880	3.1
82	16.06	35.97	2398	1199	312	887	3.1
83	16.18	36.25	2416	1208	314	894	3.1
84	16.30	36.52	2435	1217	317	901	3.2
85	16.43	36.79	2453	1226	319	908	3.2
86	16.55	37.07	2471	1236	321	914	3.2
87	16.67	37.34	2489	1245	324	921	3.2
88	16.79	37.61	2507	1254	326	928	3.3
89	16.91	37.88	2525	1263	328	934	3.3
90	17.03	38.14	2543	1271	331	941	3.3
91	17.15	38.41	2561	1280	333	947	3.3
92	17.27	38.68	2578	1289	335	954	3.4
93	17.38	38.94	2596	1298	337	961	3.4
94	17.50	39.20	2614	1307	340	967	3.4
95	17.62	39.47	2631	1316	342	973	3.4

Appendix 6

Method for the determination of Trypsin Inhibitor levels in foodstuffs.

Smith et al. (1980)

Sample preparation and extraction.

Dry products were finely ground to pass through a 100 mesh sieve. Approximately 1 g of the ground sample (weighed to an accuracy of ± 0.01 g) was briefly shaken with 50 ml 10mm NaOH and the pH of the resulting slurry was adjusted to 9.4 - 9.6 with 1M NaOH or 1M HCl. The slurry was shaken and left at 4° C overnight. After extraction the sample was shaken and diluted (D times) with water so that 1ml produced trypsin inhibition of 40 - 60 %. Dilution factors of 80, 25 and 3 were necessary for raw soya flour, medium toasted soya flour and soya-containing products respectively.

Trypsin inhibitor assay

The following additions were pipetted into a series of 10 ml tubes: (a) reagent blank: 2.0 ml deionized water; (b) standard: 2.0 ml standard trypsin solution (20 mg l^{-1} in 1mM HCl), 2.0 ml deionized water; (c)sample blank: 1.0 ml diluted extract, 1.0 ml deionized water; (d) sample: 1.0 ml diluted extract, 1.0 ml deionized water, 2.0 ml standard trypsin solution. After mixing and pre-heating to 37° C for 10 min, 5.0 ml pre-warmed Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) solution (40 mg dissolved in 1 ml dimethyl sulphoxide and diluted to 100 ml with 50 mM Tris/20mM $CaCl_2$, pH 8.2) was pipetted into each tube and mixed. After exactly 10 min incubation at 37° C, each tube recieved 1.0 ml acetic acid (30 % v/v) to stop the reaction. Standard trypsin (2.0 ml) was then added to the reagen blank (a), and sample blank (c) tubes. After filtration (no. 542 paper) or centrifugation, the absorbance of the clear solutions was measured at 410 nm.

Calculation

The change in absorbance (A_t) due to trypsin inhibition ml^{-1} diluted sample extract is $(A_b-A_a)-(A_d-A_c)$, where the superscripts refer to the tubes (a) - (d). The percentage inhibition in each sample is given by $100\ A_t/(A_b-A_a)$. If this is < 40% or >60% the assay must be repeated with a more suitable sample dilution. Since 1μg pure trypsin would give an absorbance of 0.019, the weight of pure trypsin inhibited ml^{-1} diluted sample extract is A_t/ 0.019 μg (i.e. 50 A_t / 19mg 50 ml^{-1}). From this value is calculated the trypsin inhibitor activity (TIA) in terms of mg pure trypsin inhibited g^{-1} sample as weighed (mg g^{-1}):

$$TIA = \frac{2.632 \times D \times A_t}{S}$$

Where S = sample weight. Separate moisture and nitrogen determinations will allow TIA to be expressed in terms of dry matter or protein.

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