PEA PROTEINS FOR PIGLETS :

EFFECTS ON DIGESTIVE PROCESSES

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Le Guen, M.P., 1993. White-flowered pea (*Pisum sativum*) contains 20 to 25% crude protein (Nx6.25). The pea proteins consist in globulins (60%) and albumins (25%). Because the pea albumin proteins have biological activities due to their metabolic roles in the seed, some of them are called antinutritional factors (ANFs) for monogastric animals. These are mainly protease inhibitors and lectins. The apparent ileal digestibility of N and amino acids of raw peas fed to piglets was low. The N apparent ileal digestibility of pea protein isolate consisting mainly of globulins without ANFs was 15 units higher. The addition of 3% of pea ANF concentrate decreased the N apparent ileal digestibility by 7 units. This effect is not entirely due to the alleged antinutritional activities of the protease inhibitors or the lectins. This was suggested by the results of the incorporation of another pea ANF concentrate with a higher ANF content than the former concentrate. The albumin proteins themselves were suspected to be low digestible. This was illustrated by the results of a mathematical study of the amino acid composition of ileal digesta from piglets fed a raw pea diet, showing that these amino acids would be mainly of "endogenous + bacterial" origin and partly of dietary origin and especially of albumin type. No relations, neither between N digestibility and pancreatic protease activity in the pancreatic tissue, in its secretions or in the small intestinal digesta, neither between the activities in these different sections of the gastro-intestinal tract could be established. The trypsin and chymotrypsin activity decreased in the jejunal digesta and increased in the ileo-caecal digesta upon addition of pea ANF concentrate to a pea protein isolate diet although the N apparent ileal digestibility was not affected. The enzyme activities in the pancreatic tissue was decreased with a raw pea diet compared to a pea protein isolate diet although equivalent activities were found in the pancreatic secretions. These observations suggested that the mode of action of pea protease inhibitors may depend on the demand of proteases to hydrolyse the dietary proteins in the upper intestine, and on the nutritional quality of the dietary proteins associated with these protease inhibitors.

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à la mémoire de mon
père qui rêvait d'être
agriculteur aux Pays-Bas

à mon fils et mon futur bébé

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CONTENTS

GENERAL INTRODUCTION

In the European Community (EC, 12 countries), the animal feed production amounted to 109.1 million tons (Mt) in 1991; 30.7 Mt as poultry feed (28%), 37.8 Mt as pig feed (35%) and 34.0 Mt as cattle feed (31%) (Statistiques protéagineux, 1993). In France, the feed production represented in 1991 a total of 19 Mt, divided in poultry feed (39%), pig feed (29%) and cattle feed (21%).

The plant and animal protein consumption by animals in EC in 1991 amounted to 15.8 Mt. Soya bean products provided 57.5% of them, rapeseed 9.4%, sunflower 7.3% and legume seeds 5.7%. In 1991, 11.8 Mt soya beans and 10.3 Mt soya bean meal were imported in EC. Half of the amount of soya beans came from the United States and the rest from Argentina and Brazil, while more than half of the amount of soya bean meal came from Brazil.

The production of plant and animal proteins for animal feeding in EC in 1991 amounted to 6.3 Mt. Rapeseeds, legume seeds and sunflower seeds covered respectively 23.2%, 17.9% and 10.4% of this production. This means that only about 40% of the protein consumption by animals was covered by EC production. The deficit in protein supply in EC was therefore about 60% in 1991.

To become more self-supporting in protein supply for animals, the production of European protein-rich crops and the substitution of imported soya beans by European crops were stimulated by a price-supporting policy of the EC. Legume seeds such as peas became in that respect interesting as an alternative to soya bean. Indeed the production of peas (*Pisum sativum*) increased extensively in Europe in the last years (Table 1).

Table 1. Annual production of peas in the European Community (EC) and in France

1000 tons	1982	1984	1986	1988	1990	1992
EC	*556*	*1 275*	*2 156*	*2 560*	*4 495*	*3 944*
France	*398*	*680*	*1 185*	*2 595*	*3 612*	*3 306*

from "Statistiques protéagineux" 1993

The present study is focussed on the use of peas by pigs for the following reasons. Peas (*Pisum sativum*) represent the largest proportion of the legume seeds produced in EC, and pigs are the biggest consumer of peas in EC.

White-flowered smooth peas (*Pisum sativum*) contain 20 to 25% crude protein (Nx6.25), 40 to 50% starch, 6% to 8% oligosacharides as saccharose (2 to 3%) and α-galactosides (4 to 5%), about 15% non-starch polysaccharides including 8 to 9% NDF, and about 2% lipids. Peas, as many legume seeds, contain factors called antinutritionnal factors (ANFs) because they are suspected to produce deleterious effects in animals (Burns, 1987; Birk, 1989). The best known ANFs in peas are protease inhibitors (PI) which are proteins characterized by their properties to bind serine proteases like trypsin and chymotrypsin. In plants, these ANFs act as biopesticides (Birk, 1989). Lectins are other known ANFs of protein type found in legume seeds. They have the property to bind to the carbohydrate moiety of glycoproteins of the intestinal mucosa. The α-galactosides can also cause antinutritional effects. As these are not digested in the small intestine of monogastrics, they are source of fermentation in the gut and may result in flatulence, diarrhoea and disconfort in the animals (Saini, 1989).

On a nutritional point of view, compared to soya proteins, pea proteins contain more lysine (+ 20%, LYS) but less tryptophan (-35%, TRP), threonine (-5%, THR) and sulfur amino acids (-5%, MET+CYS) (Table 2). As for the proportion of these amino acids relatively to lysine, pea proteins are less similar to the ideal protein for pigs (Wang and Fuller, 1989; MET+CYS 61%, THR 64%, TRP 20%, of lysine content) than soya proteins (Table 2).

Table 2. Content of some essential amino acids in raw pea protein and soya protein (in % of crude protein and in % of lysine content)

	% in CP		% of LYS content	
	pea	soybean	pea	soybean
LYS	*7.39*	*6.26*	*100*	*100*
MET+CYS	*2.69*	*2.84*	*36*	*45*
THR	*3.74*	*3.96*	*51*	*63*
TRP	*0.87*	*1.35*	*12*	*22*

from RPAN 1989

To become a substitute for soya bean products, peas should be as efficient as these to support growth in monogastric animals. However growing pigs or weaned piglets show sometimes lower performance when they are fed raw peas instead of soya bean meal (Grosjean *et al.*, 1986; Bengala Freire *et al.*, 1989). This was confirmed in a more recent experiment in which piglets fed a diet containing 40% raw peas had a lower growth rate than piglets fed a soya bean meal diet, although the diets were balanced for the total amount of essential amino acid (Jondreville *et al.*, 1992).

It is not clear which factor is responsible for the inefficient utilisation of peas. The proteinaceous ANFs present in peas may be one factor. When dietary PI inhibit secreted pancreatic proteases in the intestine, digestive processes may be modified. For instance, the

pancreas may be stimulated to secrete more proteases (Gertler and Nitsan, 1970; Laportes and Trémolières, 1973; Temler *et al.*, 1984) and as a result may show hypertrophy (Gertler *et al.*, 1967). Increased pancreatic secretion represents a loss of sulfur amino acids with regard to the part not reabsorbed from the intestinal lumen. This is therefore a loss of amino acids for body protein synthesis. However, with peas, hypertrophy of the pancreas is not observed in all animal species (Huisman *et al.*, 1990*a*). A literature review on the exocrine pancreas (Chapter II) showed that indeed pancreatic functioning is submitted to many sources of variation. The lectins could also be considered as they can increase mucus secretion and desquamation rate of the intestinal mucosa and decrease intestinal absorption efficiency (Kik, 1991). However no adsorption of pea lectins on intestinal mucosa has been observed in rats (Aubry and Boucrot, 1986) and in piglets (Bertrand *et al.*, 1988), but binding of pea lectins to intestinal disaccharidases and proteases has been observed in rats by Jindal *et al.* (1982).

The hypothesis that PI would be partly responsible for the inefficient utilisation of raw peas by pigs has been derived from experiments in which several batches of peas differing by their level of PI activities were tested (Huisman, 1989; Leterme *et al.*, 1989). However PI activity may not be the only source of variation in performance of pigs fed different batches of peas. This was shown in the present thesis in which low apparent ileal N digestibility coefficients were found for raw peas with a high as well as with a low PI activity, fed to piglets (Chapter III).

These low apparent ileal N digestibility coefficients were the base of the present study. Various experiments were designed to find an explanation for these results. The protocol chosen was to isolate the main components in peas according to Guéguen (1983): a protein isolate without PI and lectins, a protein concentrate rich in PI and lectins which was called ANF concentrate, and a carbohydrate isolate. Diets based on isolated pea proteins without ANFs were made. The effects of adding ANF concentrate or pea carbohydrates to these pea protein isolate diets were studied. The reactions of the piglets with regard to N apparent ileal digestibility with each of these diets were evaluated (Chapter III). The results concerning pea carbohydrate addition were reported in Huisman *et al.* (1990*b*). In addition to the nutritional evaluation, the proportions of endogenous N in the ileal digesta from piglets fed raw peas or pea protein isolate were evaluated using a mathematical model developped by Duvaux *et al.* (1990) (Chapter IV). This was based on the amino acid compositions of reference proteins and of ileal digesta.

The reactions of piglets were studied also with regard to pancreatic enzymes in relation to PI intake. Trypsin and chymotrypsin activities were measured in the pancreatic tissue and its secretions (Chapter VI) but also in different parts of the gastrointestinal tract (Chapter V). The measurements of these physiological parameters was to get insight into the mode of action of PI and into the interaction between pancreatic enzyme activities and ileal digestion of N.

In the general discussion (Chapter VII) other factors such as antigenicity of pea proteins or non starch polysaccharides were discussed to fully explain the low digestibility of raw peas measured in the first experiments (Chapter III). A general model for mode of action of pea PI, taking into account dietary variations, is also proposed (Chapter VII).

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Chapter II

ROLE OF THE PANCREAS IN THE DIGESTION OF PROTEINS:

LITERATURE REVIEW

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ABSTRACT

Raw peas exhibit a low apparent digestibility when fed to young pigs. They are know to contain chemical substances called antinutritional factors (ANFs) which are partly responsible for the low nutritional value of raw peas. Among ANFs, a large group of proteins, called protease inhibitors is present. A variable relation between protease inhibitors and pancreatic function has been reported in many studies. As the pancreas plays a major role in digestive processes, it is important to understand the effects of pea ANFs on the pancreas of piglets in order to pin-point the low nutritional value of raw pea. A literature review on the physiology of the exocrine pancreas and the effects of protease inhibitors on pancreatic function was prepared.

INTRODUCTION

Growth performance and digestibility values are sometimes unsatisfactory when piglets are fed diets containing raw peas (Grosjean, 1985; Grosjean and Gatel, 1986; Savage and Deo, 1989; Matre *et al.*, 1990; Jondreville *et al.*, 1991).

Some pea proteins exhibit antinutritional properties which can affect digestive and metabolic processes in animals and in humans. They are called antinutritional factors (ANFs). One class of pea ANFs which have the property to bind trypsin and chymotrypsin, is generally referred to as protease inhibitors (PI). Another class of ANFs generally encountered in pea seeds, is also protein in nature with an ability to bind carbohydrates of the glycoproteins of the small intestinal brush border membrane and the lectins. These latter proteins, called lectins, appear to have no deleterious effects on piglets as reported by Aubry and Boucrot (1986) and Bertrand *et al.* (1988), although they were shown to damage the gutwall in rats (Jindal *et al.*, 1982).

Protease inhibitors bind pancreatic proteases in the small intestine. It is thought that the result of this enzyme inactivation is a decreased digestibility of dietary proteins and therefore decreased growth performance of the animals. However Liener *et al.* (1949) showed that addition of soybean PI to a diet based on predigested proteins fed to rats still reduced their growth rate. These effects may originate in the regulation mechanisms of the pancreas. From studies carried out on soybean PI, it was shown that the pancreas reacts to protease inactivation in the small intestine (Liener, 1979; Liener and Kakade, 1980; Gallaher and Schneeman, 1986; Birk, 1989). The physiological responses observed upon feeding PI were enlarged pancreas in rats (Gertler *et al.*, 1967; Kakade *et al.*, 1973; Roy and Schneeman, 1981; Grant *et al.*, 1992; Pusztai *et al.*, 1992) and in chickens (Gertler and Nitsan, 1970), hypertrophy of the pancreas in rats (Green *et al.*, 1986), and increased secretion of pancreatic enzymes in chickens (Gertler and Nitsan, 1970). However these physiological effects on the

pancreas are not observed for every animal species (Struthers *et al.*, 1983*a*; Rascon *et al.*, 1985; Schneeman and Gallaher, 1986). No enlargment of the pancreas or no increase of its protease content or its secretions were observed in guinea-pigs (Hasdai *et al.*, 1989), piglets (Yen *et al.*, 1977; Huisman *et al.*, 1990*a*) and calves (Guilloteau *et al.*, 1986; Le Dréan *et al.*, 1992) upon feeding diets containing PI.

To understand the complex role of the pancreas in the digestion of diets rich in PI, such as raw pea diets, by piglets, physiology and regulation factors of the exocrine pancreas were reviewed.

MORPHOLOGY OF THE PANCREAS

In the pig, the pancreas is located under the lumbar area. It is crossed by the portal vein but no exchange occurs between the pancreas and this vein. It represents 0.17 - 0.18% of the live weight in 8 week-old piglets.

The pancreas is a dual digestive gland that secretes, on the one hand hormones in the blood (*endocrine* part) and on the other digestive fluids in the intestinal lumen (*exocrine* part). The endocrine and the exocrine parts are not physically separated in the organ (Figure 1).

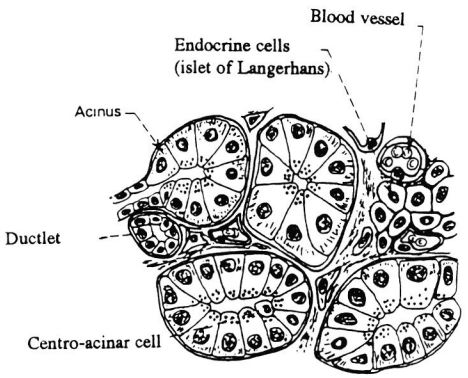

Figure 1. General view (x1500) of a histological section of a pancreas from mammals. (adapted from Rieutort, 1982)

The exocrine part of the pancreas is the most voluminous. It represents about 99% of the total mass in the rat (Rieutort, 1982). The endocrine pancreas consists of the islets of Langerhans,

clusters of cells scattered throughout the exocrine pancreas. Four hormones secreted by the endocrine pancreas directly into the blood have been detected (Rieutort, 1982):

- glucagon: a 29-amino-acids polypeptide. It induces an increase in blood glucose by stimulating glycogenolysis in the liver.
- insulin: a two polypeptide chains. It acts to reduce the level of blood glucose.
- somatostatin: a 14-amino-acids polypeptide. It inhibits the secretion of the somatrope hormone from the hypothalamus.
- pancreatic polypeptide: its complete functions are unknown. Among other functions, it would inhibit the exocrine pancreas.

Due to its secretory activity, the protein turnover in the pancreas is very high. In pigs of 30 kg body weight, the average proportion of protein mass which is replaced each day in the pancreas has been found to equal 111% per day, as compared to 50% in the small intestine and 8% in the heart (Simon *et al.*, 1978). The RNA content of each pancreatic cell is one of the highest of the body (Rieutort, 1982).

THE EXOCRINE PANCREAS

1. Structure and Physiology

1.1. Structure

The exocrine pancreas is divided into two functional parts - acinar cells and duct cells (Figure 2).

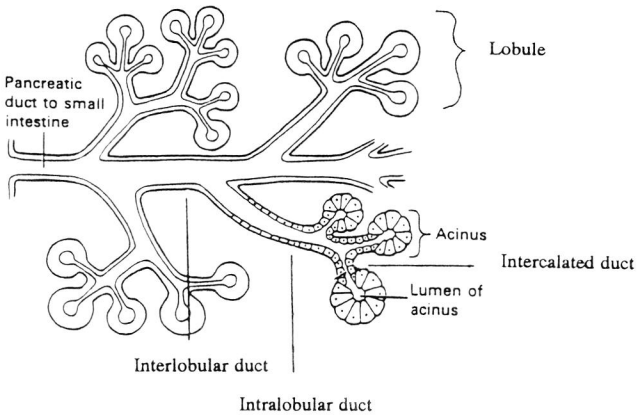

Figure 2. Schematic representation of the exocrine pancreas (adapted from Darnell *et al.*, 1986)

Six to eight acinar cells form a functional unit called the acinus. These units are sometimes lined by centro-acinar cells. The fluid formed by the acinar cells is released into the lumen of the acinus, transported into the intercalated duct, then into the intralobular duct (merging of intercalated ducts). This structure of acini and ducts is called a lobule. The lobules are connected by interlobular ducts to the main pancreatic duct that enters the duodenum (Rieutort, 1989).

1.2. Physiological role

The pancreatic fluid secreted by the exocrine pancreas into the duodenum is the most important digestive fluid. It contains enzymes and bicarbonates, the former being secreted by the acinar cells, the latter by the centro-acinar cells and the duct cells (Schulz, 1987). The enzymatic composition of the fluid has been studied in pigs by Marchis-Mouren (1965) and Marchis-Mouren *et al.* (1961) by means of chromatography. Most of the enzymes are secreted in an inactive form, as precursors called zymogens. In this form, the enzymes do not degrade the pancreatic tissue in which they are produced.

The proteases are classified into two categories :

- endopeptidases: they hydrolyse peptide bonds inside the polypeptide chains. Examples of endopeptidases are : trypsin, chymotrypsin, elastase and collagenase.
- exopeptidases: they hydrolyse peptide bonds at the carboxyl-end of the polypeptide chains. Examples of exopeptidases are carboxypeptidases A and B.

Amylase and lipase are the other main enzymes also found in the pancreatic juice. Elastase and collagenase also present in pancreatic fluid hydrolyse elastic fibres and collagen respectively, allowing accessibility to the other endopeptidases (Simoes Nunes, 1982). The other components of the fluid are water and electrolytes. The major role of bicarbonate secretion is to ensure a correct intraluminal pH for the enzymes to exert their activity.

Trypsin hydrolyses peptide bonds where the carboxyl is contributed by an amino acid with a basic side chain (lysine and arginine). Pig pancreatic juice contains two types of trypsins: an anionic trypsin and a cationic trypsin (Kidders and Manners, 1978)

Chymotrypsin hydrolyses peptide bonds where the carboxyl group is derived from a neutral aromatic amino acid such as phenylalanine, tyrosine and tryptophan (chymotrypsin A), or derived from leucine (chymotrypsin B) or from glutamic acid and methionine (chymotrypsin C).

The exopeptidases, complementary to the endopeptidases, act at the carboxyl end of polypeptides, on bonds of the neutral aromatic amino acids (carboxydase A) and the basic amino acids (carboxydase B).

Peptide bonds involving proline amino acid are not hydrolysed by pancreatic proteases but by specific peptidase of the enterocytes of the brushborder membrane (Simoes Nunes, 1982).

1.3. The acinar cells

a. Cellular polarity (Figure 3)

The acinar cells of the pancreas have a strong polarity for their structure as well as their physiology, meaning a specific pathway taken by newly synthesized secretory proteins (Darnell *et al.*, 1986). The cells have the shape of a pyramid. The large base (basal pole) is orientated towards the blood vessels for nutritional supplies. The narrow top (apical pole) is turned toward the lumen of the acinus. The apical zone is full of granules containing zymogens (1μm $\varnothing$). During fasting, the concentration of granules is high in this area. This large reserve capacity allows the pancreas to secrete a large amount of protease in a short postprandial time (Corring, 1980). The basal zone contains the nucleus, the mitochondries, the rough endoplasmic reticulum and the Golgi sacs. The physiological polarisation of the acinar cells can be represented by an arrow from the basal to the apical pole, indicating the pathway of the secretory proteins. The large cavities of the rough endoplasmic reticulum are orientated according to this direction. The rough endoplasmic reticulum represents about 60% of the cytoplasm volume in the acinar cells (Rieutort, 1982).

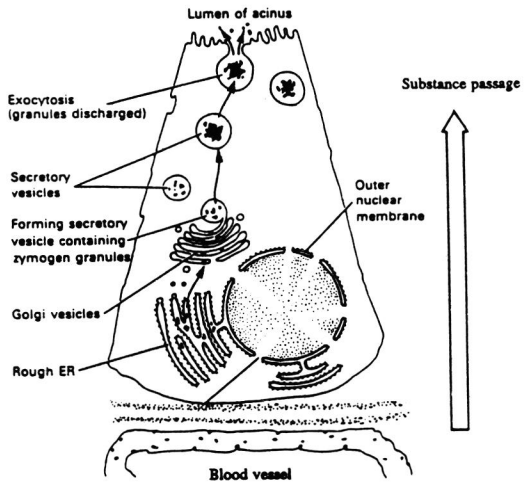

Figure 3. Schematic representation of the acinar cell with indication of its physiological polarity (adapted from Darnell *et al.*, 1986; Rieutort, 1982)

b. Pathway of secretion (Figure 3)

Immediately after synthesis, the secretory proteins are found in the lumen of the rough endoplasmic reticulum. The transfer vesicles which have budded off the rough ER, fuse with the membranes of the Golgi complex. The vesicles budding off the Golgi sacs contain the zymogens. The exocytosis of the secretory vesicles is triggered by hormonal and/or neural stimulation. The zymogens are then released into the acinus lumen, then into the pancreatic ducts and finally into the intestine where they are transformed into active digestive enzymes (Darnell *et al.*, 1986).

1.4. Pancreatic trypsin and chymotrypsin in the intestine

a. Zymogens activation

The zymogens in the intestine undergo specific irreversible proteolytic cleavages (Darnell *et al.*, 1986). The activations of the zymogens are dependent upon each other. It starts with trypsinogen activated by enterokinase present in the intestinal juice. The active trypsin can then activate trypsinogen, chymotrypsinogen, procarboxypeptidases and proelastase (Keller, 1968; Rovery, 1988) (Figure 4).

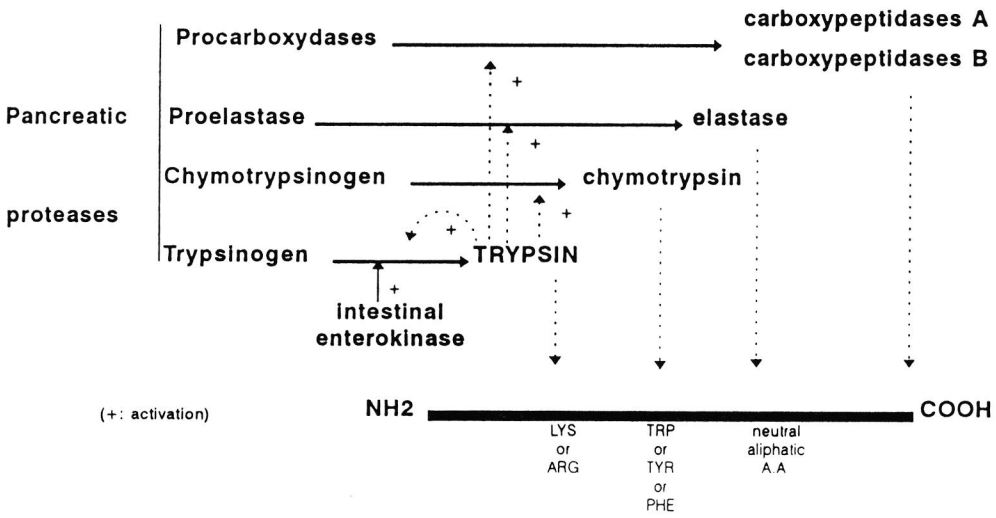

Figure 4. Activation and specificity of the main pancreatic proteases in the intestine of monogastric animals (+: indicates activation; NH2--COOH: polypeptidic chain)

b. Substrate binding

The binding of an enzyme to its substrate occurs at a specific site. For chymotrypsin, this site is an hydrophobic cleft, consistent with the substrate amino acids specificity of this enzyme. For trypsin, the amino acids lining the site have negatively charged side chains, thus facilitating the binding with only positively charged residues (lysine, arginine).

Most water-soluble proteins are globular. They are folded so that most of the hydrophobic amino acids are in the interior part of the molecule, away from the water. If such proteins arrive in the intestine in their native form, they are not accessible to chymotrypsin for hydrolysis. Normally stomach acids partly denature ingested proteins, and pepsin degrades them partially before they enter the duodenum. In that way the proteins are partly unfolded and can be reached for hydrolysis by the enzymes.

2. Stimulants of pancreatic secretion

In the absence of food in the stomach and small intestines, there is a basal pancreatic secretion which accounts for about 10 to 30% of the maximal secretory rates (Solomon, 1987). Further secretion is stimulated as soon as the meal is consumed.

2.1. Postprandial stimulation of the exocrine pancreas

The secretion of pancreatic juice of an appropriate composition and amount for efficient neutralization of gastric acid and digestion of macronutrients is the result of interaction of various stimulants (Corring, 1977; Corring *et al.*, 1989). The stimulation is initiated and maintained during the first 5 hours by action of dietary nutrients at different levels of the proximal small intestine (Corring *et al.*, 1989).

The first phase of the stimulation of secretion is called the cephalic phase. Stimuli such as sight, smell, taste and act of eating food result in increased secretion (Corring *et al.*, 1972).

The stomach also takes part in the stimulation (gastric phase). As the animal starts eating, the volume of pancreatic juice or the concentration of proteins in the juice increases, resulting in a higher amount of enzymes secreted in the duodenum (Corring *et al.*, 1972). The distension of the stomach due to the meal would lead to subsequent reflex stimulation of the pancreas (Cargill and Wormsley, 1979).

Almost simultaneous to the gastric phase is the intestinal phase of pancreatic secretion. The initiation of it is largely due to the gastric acid and intragastric digestion products (peptides, amino acids, fatty acids, monoglycerides) coming into the duodenum. The gastric acid and the digestion products are partly responsible for postprandial secretion of pancreatic bicarbonate and enzymes specific for the digestion of the nutrients present in the lumen (Solomon, 1987).

In man, alterations of gastric digestion or emptying lead to abnormal postprandial pancreatic enzyme secretion (Mayer *et al.*, 1982). The intragastric digestion and stomach emptying therefore seem important in determining the pattern and magnitude of the intestinal phase of the pancreatic secretion.

2.2. Stomach emptying and influence on pancreas

The length of stay of the meal in the gastric lumen have consequences on the intensity of dietary nutrients hydrolysis in the stomach, and on the amount of digestion products entering the duodenum to stimulate the pancreatic secretion of enzymes.

Stomach emptying is the result of two positive forces (pressure in the stomach and propulsion of the antral part) and two negative forces (passage resistance due to the pylorus and to pressure in the duodenum).

The evacuation from the stomach starts at the beginning of the meal. Its rate is very high during the first 30 minutes (Cuber and Laplace, 1979). It corresponds to the liquid phase being evacuated by help of the stomach pressure. The liquid emptying can be delayed by certain types of dietary fibres, especially the hydrophilic types (Low, 1989).

The second phase, when the evacuation rate decreases and stays steady (Cuber and Laplace, 1979), corresponds to the reduction of large particles. They are retained much longer in the stomach than the liquid phase; the bigger the particle size, the longer the retention. The solid phase is pushed by antral contractions. The particles too large to go through the pylorus are pushed back in the centre of the stomach. The intensity of these contractions is related more to the particle size than the stomach fullness (Laplace *et al.*, 1986). The second phase of the emptying kinetics is thus relatively independent of the size of the meal.

The length of stay of the meal in the gastric lumen also depends on several other factors. The main factors are acidity, osmolarity, levels of fatty acids and various amino acids in the chyme entering the duodenum where the receptors for these factors are located (Rérat and Corring, 1991).

The pH in the stomach is the result of the acidic juice released by the stomach mucosa and the buffering capacity of the diet. The pH is not constant during the digestion. The secretion of juice from the gastric mucosa, mainly acid and pepsinogen, starts immediately at the meal consumption. The pH reaches a peak 1 to 2 hours later, and stays at that level during 4 or 5 hours (Laplace *et al.*, 1986). But the pattern of pH changes can be affected by dietary factors (Lawrence, 1972). As acidic chyme enters the duodenum from the stomach, the duodenal pH decreases and the pylorus closes itself. The acidity simultaneously stimulates the pancreas to secrete a bicarbonate solution which neutralizes the gastric acid, raises the pH and allows the pylorus to open again.

In dogs, a pH 4.5 for the chyme entering the duodenum seems to be a threshold level above which the bicarbonate secretion from the pancreas is weak (Grossman and Konturek, 1974). In other species, the available information on the regulation of pancreatic secretion by acid in the intestine does not allow final conclusions (Solomon, 1987).

2.3. Dietary stimulants of pancreatic secretion

a. *Carbohydrates*

The amount and type of dietary fibre given to pigs affect the volume of pancreatic juice, and sometimes the protease activity (Low, 1989). As the amount of fibre increases in pig diets, the volume of pancreatic juice and the electrolyte output increase according to studies of Partridge *et al.* (1982), Zebrowska and Low (1987) and Langlois *et al.* (1987). The increased electrolyte output may be due to the higher volume of acid secreted by the stomach (Low 1989). The output of proteases was unchanged in the studies of Partridge *et al.* (1982), Zebrowska and Low (1987) but increased in a study of Langlois *et al.* (1987). The stimulation of pancreatic enzyme secretion under some circumstances may be related to an inhibition of pancreatic enzyme activity by dietary fibres, depending on their sources (Low, 1989).
Intravenous glucose injection in pigs limits pancreatic juice volume and total protein output (Simoes Nunes and Corring, 1980).

b. *Amino acids, peptides, proteins*

The dietary stimulants of proteolytic enzymes secretion from the pancreas are mainly their own substrates or by-products such as amino acids, peptides, proteins.
In contrast to intact proteins, amino acids and peptides solutions introduced in the proximal small intestine of dogs are efficient stimulants of pancreatic enzyme secretion (Meyer and Kelly, 1976). Only some amino acids such as leucine, phenylalanine and tryptophan are effective stimulants in dogs (Solomon, 1987). In human also, only some amino acids such as phenylalanine, methionine or valine, stimulate pancreatic secretion when perfused in the intestine (Solomon, 1987). In contrast to human and dogs, intestinal perfusion of amino acids or completely hydrolysed casein does not stimulate pancreatic protein secretion in rats but perfusion of intact casein doubles the amount of pancreatic protein secreted (Schneeman *et al.*, 1977; Schneeman, 1982; Green and Miyasaka, 1983). The synthesis of chymotrypsin in the pancreas can even decrease in rats when amino acids are used in the diet as the only nitrogen source (Johnson *et al.*, 1977).

The effects of intravenous administration of amino acids on pancreatic secretion are confusing. In only a few studies an effect was observed. Among various amino acids administered intravenously to chicks, phenylalanine stimulated pancreatic proteases secretion (Yang *et al.*, 1989b). In dogs however, intravenous amino acids do not affect pancreatic secretion (Stabile *et al.*, 1984).

Oligopeptides and large peptides, being the main fractions of protein hydrolysis in the stomach (Rérat, 1981), may be more important than amino acids in initiating the intestinal phase of pancreatic secretion in all animal species as amino acids do not accumulate in the intestine during digestion of a meal (Schneeman, 1982). Di- or tripeptides containing phenylalanine or tryptophan are effective intraluminal stimulants of pancreatic proteases secretion in dogs (Meyer and Kelly, 1976; Meyer *et al.*, 1976).

The type of dietary proteins itself may influence the characteristics of pancreatic secretion.
In rats, the content and synthesis of chymotrypsinogen in the pancreas can be affected by the sources of the ingested proteins, eg. zein, fish, gluten or casein (Johnson *et al.*, 1977), or the pancreatic secretions by the proteins infused in the duodenum (Berger and Schneeman, 1986).
In calves, the pancreatic juice volume, protein content and enzyme activities were lower with a soybean protein diet than with a milk protein diet, both at the same level of crude protein (Gorill and Thomas, 1967).
In pigs, the volume of pancreatic juice was lower but the chymotrypsin specific activity was higher with rapeseed protein isolate than with casein in the diet (Valette *et al.*, 1990).
The quality of the proteins themselves would be partly responsible for the different pancreatic responses observed. When poor quality proteins either due to their difficult digestion or their amino acid deficiency are fed to rats, the pancreas would be unable to modify its secretion if required (Schneeman et Gallaher, 1986).
The role of specific proteins as protease inhibitors is discussed further in this review.

The quantity of protein in the diet is also a factor of variation of pancreatic response. As the quantity of nitrogen orally ingested by pigs or rats increased, the specific and total protease activities increase both in the pancreatic tissue and juice (Corring and Saucier, 1972; Schick *et al.*, 1984). The adaptation to a new quantity of protein can be very fast, as fast as 20 min (Johnson *et al.*, 1977), or 30 min (Corring, 1980) in rats, thus preceding changes in tissue levels of enzyme, or 24 hours (Dagorn and Lahaie, 1981). The adaptation to dietary protein changes is stabilized after 5 to 7 days on a new diet (Ben Abdeljlil and Desnuelle, 1964).
Low protein diets (3% cereal protein) fed to rats during 21 days result in decrease of volume and enzyme activity in pancreatic juice determined over 48 hours (Kheroua and Belleville,

1981). The protein content, mitotic ability and enzyme activity in the pancreas were also decreased. Amino acids deficiency was supposed to be the main inhibitor (Kheroua and Belleville, 1981).

Dietary protein deprivation results in a loss of zymogen granules, shrinking and atrophy of the acinar cells and a reduction in the amount and duration of enzyme secretion from the pancreas (Rérat and Corring, 1991). The effects appear in the composition of pancreatic juice with a considerable delay in time because of the presence of large numbers of preformed zymogen granules (Malfertheiner *et al.*, 1988). In case of complete protein deprivation, the protein synthetic activity of the acinar cell is directed to an almost exclusive production of a group of anionic proteases (Schick *et al.*, 1984).

The adaptation for the first time to the new diet is quick as mentionned earlier. It occurs without any contact between the nutrients and the pancreas, and before any intestinal absorption of hydrolysis products, suggesting an active involvement of the stomach via gastrin release during the cephalic and gastric phases (Solomon, 1987), and (or) of the intestinal mucosa via a messenger (Corring *et al.*, 1989). Dick and Felber (1975) suggested that the variation in the size of the intestinal pools of hydrolysis products might be the sensor for pancreatic adaptation and might be at the origin of a process of messenger release.

The mechanisms that mediate the pancreatic response to dietary changes or intestinal infusion of amino acids appeared to be confined to the duodenum and jejunum, in dogs (Konturek *et al.*, 1972). In pigs, however, the adaptation of the pancreas to modified dietary protein content would not be limited to the proximal small intestine, as the increased pancreatic protease secretion resulting from an increased protein content of the diet, occurred even when the first three meters post-pylorus were shunted (Simoes Nunes, 1982; Corring *et al.*, 1989).

3. Regulators of pancreatic secretion

3.1. Trypsin activity as a regulator: feedback regulation

Numerous studies have demonstrated that pancreatic enzymes exert a feedback control of pancreatic exocrine secretion in several animal species. In pigs and rats, the control operates at the level of the duodenum (Corring, 1974; Schneeman and Lyman, 1975).

Removal of pancreatic juice from the intestine results in a large increase in pancreatic enzyme secretion in rats (Green and Lyman, 1972), and in volume of juice and amount of proteins secreted by the pancreas of pigs (Corring, 1974). By reintroducing the juice in the intestine, the secretion comes back quickly to its initial level (Corring, 1973 and 1974) (Figure 5), which is not the case when only the bicarbonate solution or the enzyme-free juice is

reintroduced. Therefore, it is only the protein part of the pancreatic juice that is involved in the observed feedback mechanism (Corring, 1974).

The feedback regulation is based on the enzymatic activity of trypsin and chymotrypsin, not on the molecule itself (Iwai *et al.*, 1988; Fushiki and Iwai, 1989). Therefore the inhibition of trypsin and (or) chymotrypsin activity by soybean trypsin inhibitors can result in a large increase in pancreatic enzyme output in rats (Laporte and Trémolières, 1973; Temler *et al.*, 1984) and in pigs (Fukuoka *et al.*, 1986), as does the diversion of pancreatic juice.

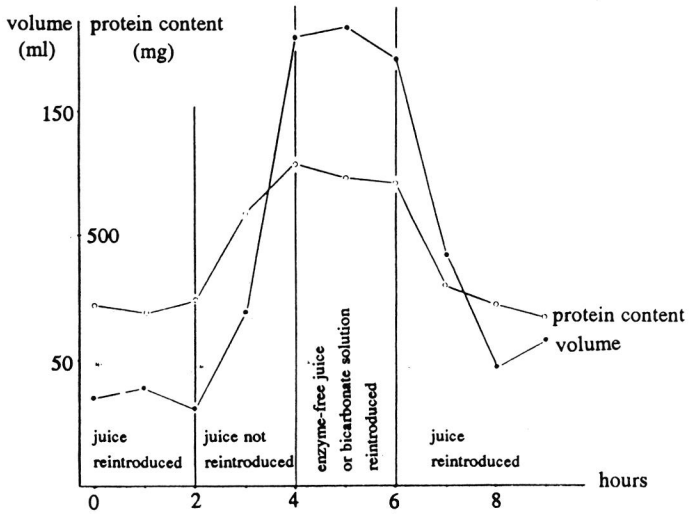

Figure 5. Effect of diversion and reintroduction of pancreatic juice in the duodenum in pigs. Effect of intraduodenal infusion of bicarbonate or enzyme-free solutions (adapted from Corring, 1974)

Mediation of the effects of a meal on pancreatic secretion is ascribed to neurotransmitters and to hormones. But the function of many regulatory peptides has not been fully evaluated.

3.2. Neural regulators

There is strong evidence that during the cephalic, gastric and intestinal phases of pancreatic stimulation, intrapancreatic neurons are activated, via central input and vagovagal reflexes (Solomon, 1987). Acetylcholine released by these neurons acts directly on receptors, on acinar and duct cells to elicit enzyme and bicarbonate secretion (Solomon, 1987). Vasoactive intestinal polypeptide and gastrin releasing peptide nerves are likely to be involved in the neural stimulation of the exocrine pancreas (Holst, 1985).

The effect of intraduodenal amino acids on the pancreas would be mediated at least in part by the neural mechanism (Singer, 1983).

3.3. Hormonal regulators

Secretin and cholecystokinin are considered to be the major hormonal regulators of the exocrine pancreatic response to meals.

a. *Secretin*

Secretin is a 27-amino-acid peptide secreted by open cells of the proximal small intestine. According to Holst (1985), it would be secreted from the upper jejunal mucosa but according to Kidder and Manners (1978) it would predominantly originate from the proximal part of the duodenum. In pigs, the release of secretin is stimulated by HCl, which would be the transmitting link between emptying of gastric acid into the duodenum and pancreatic bicarbonate secretion (Holst, 1985). According to Holst (1985), dietary nutrients or mixed food preparations would not stimulate the secretion of secretin. Corring *et al.* (1985) observed that when the pancreatic juice secreted by pigs was removed, the plasma level of secretin increased by 150%. As the ingestion of soybean trypsin inhibitors by pigs results also in an increase of secretin level in the blood (Corring *et al.*, 1986), gastric acid is not the only stimulant but also the protein part of the pancreatic juice.
An increase of dietary fibre may also increase circulating level of secretin (Langlois *et al.*, 1987), perhaps because of the higher amount of gastric acid.
Exogenous secretin administered intravenously increased total pancreatic protein secretion in rats (Haarstad and Petersen, 1988). In pigs, secretin stimulates the flow rate (Corring, 1974; Hermann and Cier, 1979) and the concentration of bicarbonates in the pancreatic juice (Hermann and Cier, 1979; Corring and Chayvialle, 1982). The pig pancreas is extremely sensitive to secretin (Holst, 1985).
In a few animal species, secretin induces acetylcholine neural stimulation (Solomon, 1987).

b. *Cholecystokinin*

Cholecystokinin (CCK) is a gut hormone, located in neuroendocrine cells of the proximal small intestine and is not exclusively confined to the duodenal mucosa in the pig (Kidder and Manners, 1978). It is released into the blood when dietary proteins are ingested by rats (Liddle *et al.*, 1984) or amino acids ingested by chicks (Yang *et al.*, 1989a). In chicks, plasma CCK level can increased 30 minutes after tube-feeding of an amino acid mixture, or 60 minutes after tube-feeding a soy-protein diet (Yang *et al.*, 1989a).

Multiple molecular forms of CCK, ranging in size from 4 to 58 amino acids, have been identified in the intestine and plasma. CCK8 and CCK33 are the most abundant forms of CCK in plasma. Both are biological active peptides.

CCK, by linkage to specific receptors on the pancreatic cells stimulates the pancreas to secrete more pancreatic enzymes. When CCK is administered intravenously to chicks, with or without amino acids, the enzyme secretion is extensively increased as compared to amino acids alone (Yang *et al.*, 1989b). In rats, in addition to an increased secretion of pancreatic enzymes, exogenous CCK induces an increase of the mass, the total enzyme and DNA content and the number of cells in the pancreas (Miazza and Loizeau, 1985). The magnitude of the trophic effect depends on the dose administered. According to Malfertheiner *et al.* (1988), the pancreatic response of rats to released CCK would be regulated by the protein content and quality of the diets; the pancreas would not be able to supply more enzyme under a CCK stimulation, in case of a protein deficiency or a poor quality protein.

The release of CCK would be related with trypsin and chymotrypsin activity in the intestine. The mechanism underlying this relation (trypsin ↔ CCK) is not yet fully understood. Fushiki and Iwai (1989) developed a model for this regulatory mechanism. The pancreas would release a monitor-peptide into the duodenum. During the preprandial phase, this monitor would be hydrolysed and thus inactivated, by trypsin and chymotrypsin in the duodenum. When dietary proteins come into the lumen, trypsin and chymotrypsin would rather cleave these proteins than the monitor-peptide, leaving it active to stimulate the release of CCK into the circulation.

However there would be another peptide originating from the small intestine involved in the regulation, as diversion of pancreatic juice also induces a hypersecretion of pancreatic proteins. Lu *et al.* (1989) found in rats a trypsin-sensitive peptide secreted by the proximal small intestine that stimulates the release of CCK into circulation.

c. *Inhibitory hormones*

Ingestion of a meal could stimulate the release of inhibitory hormones or activate inhibitory reflexes that act directly on the pancreas or on the release of stimulatory hormones such as secretin or CCK, or on the release of intrapancreatic neurotransmitters such as acetylcholine and vasoactive intestinal polypeptide (Solomon, 1987).

Three hormones - glucagon, somatostatin, pancreatic polypeptide - secreted by the endocrine pancreas are potential inhibitors of postprandial pancreatic secretion (Holst, 1985).

In pigs, exogenous pancreatic polypeptide has been shown to induce a significant inhibition of pancreatic juice protein concentration and output without changes in volume and bicarbonate (Langlois *et al.*, 1989). In dogs, these characteristics of pancreatic juice were significantly

reduced after injection of PP (Lonovics *et al.*, 1981; Shiratori *et al.*, 1988). Glucagon injected intravenously in pigs induced a sharp decrease of the secretion of pancreatic volume and proteins (Corring, 1974).

MEASUREMENT OF PANCREATIC ACTIVITY

Evaluation of *in vivo* pancreatic adaptation to dietary changes depends on the selection of products that will reveal this adaptation, on the selection of measurements and on the selection of assay procedures for protease activity.

1. Products

Three main categories of products providing data on the pancreas exist. These include the organ itself, its secretions and the digesta mixed with the secretions.

The measurement of the enzyme activity in the pancreas is a way to assess the pancreatic adaptation to an experimental feed. It requires the slaughter of the animals at a determined post-prandial time, and a rapid freezing of the organ. However this measurement is not adequate when an evaluation of the short-term effect of a meal is required for example, to know the total amount of pancreatic enzyme produced by the pancreas for this meal. The amount of enzyme activity in the pancreas a few hours after the meal is the result of secreted enzyme activity via the juice and of newly synthesized enzyme activity. As the amount secreted in the juice is not known in that way, the total amount produced for the meal is then unknown.

As for pancreatic juice, it can be collected via a cannula, either directly placed in the pancreatic duct (Corring *et al.*, 1972) or in an isolated segment of the duodenum where the pancreatic duct opens (Zebrowska *et al.*, 1983; Hee *et al.*, 1988). In any case, the pancreatic juice has to be returned in order to maintain a normal functional pancreas without interfering with the feedback mechanism. Pancreatic secretion can also be studied in intact isolated pancreas, kept *in vitro* and perfused with an oxygenated medium (Jensen *et al.*, 1978).

As for the other type of products, the digesta, they can be collected from different parts of the small intestine, from cannulated animals or at slaughter of the animals. The collection should be made in relation to the feeding time.

Small amounts of pancreatic enzymes can also be found in faeces (Nitsan and Liener, 1976*b*; Struthers *et al.*, 1983). In blood plasma or serum small enzyme molecules, as lipase, can be detected (Frobish *et al.*, 1971).

2. Measurements

According to the products selected for data on pancreas, different measurements are possible. In the pancreatic tissue, in addition to the weight of the organ, biochemical parameters can be determined:

- enzyme activity,
- protein content,
- DNA as a measure of cell number,
- protein/DNA as an index of cell size,
- RNA content,
- enzymatic mRNA concentration (Corring *et al.*, 1989),
- protein biosynthesis, by determining the protein-bound radioactivity in the pancreas after infusion of ^{75}selenomethionine for example, in the pig (Haarstad and Petersen, 1988).

Histological and ultrastructural examinations can also be made, giving informations about:

- cellular proliferation (nodular hyperplasia or not),
- acinar adenoma or not,
- nuclear density and size,
- mitotic index (number of mitotic figures per 1000 nuclei),
- granules size and density.

The most common measurements are volume, protein content and enzyme activity in pancreatic juice, and enzyme activity in digesta.

3. Protease activity

3.1. Assay procedures

The most common method used to assay protease activity is based on the use of a substrate specific for the enzyme under consideration (Schwert and Takenaka, 1955; Bundy, 1963; Erlanger *et al.*, 1966). The activated enzyme of the product is placed in contact with such a substrate that contains arginine (for trypsine) or tyrosine (for chymotrypsine). The enzyme splits the substrate at the carboxyl end of its specific amino acid, leaving an ionized carboxyl end -COO^-. The activity can be measured by titration, or by spectrophotometry if the selected substrate was one of those capable of releasing a coloured product directly upon hydrolysis. The activity is then expressed by the quantity (μmol) of substrate hydrolysed per minute under assay conditions.

3.2. Variations

Direct comparison of enzyme activity data in the literature is difficult due to a number of factors involved.

The sampling and storage conditions of pancreatic juice are important elements in that respect. Chymotrypsin activity for example decreased after one day storage at 4°C (Makkink *et al.*, 1990).

The time interval between last feed intake and slaughter is an important factor for enzyme activity in the pancreas (Nitsan and Liener, 1976*b*; Crass *et al.*, 1987). If the pancreas is collected a few hours after the meal, the enzyme activity is then the resultant of secretion and biosynthesis of the enzyme. However, in a fasted state, the result reflects the adaptation of the pancreas to a dietary variation.

A third element in the comparison of literature data is the substrate used when measuring trypsin or chymotrypsin activity. The ratios of chymotrypsin/trypsin activity can be as high as 5 to 6 (Aumaître, 1971; Corring *et al.*, 1978, 1984) or 15 to 20 (Gertler and Nitsan, 1970), when measured by titrimetry using the substrates benzoyl-L-arginine-ethylester (BAEE) for trypsin and N-acetyl-L-tyrosine-ethylester (ATEE) for chymotrypsin. However these ratios are between 0.1 and 0.5 when measured by spectrophotometry using the substrates p-toluenesulfonyl-L-arginine-methylester (TAME) and benzoyl-L-tyrosine-ethylester (BTEE) (Yen *et al.*, 1977; Partridge *et al.*, 1982; Zebrowska *et al.*, 1983).

For the measurement of trypsin activity in the chyme, there are several other factors to be considered. The measurement is sometimes carried out without activation of the zymogen, assuming it to have already occurred in the intestine before collection. However, the extent of activation depend on several factors (Scheele and Palade, 1975). The recovery of activity in the chyme is another important aspect. The loss of activity and its variation during the time course in the small intestine is unknown. Moreover trypsin binds avidly to small food particles, but the extent of this phenomenon differs according to the diet.

PROTEASE INHIBITORS AND PANCREAS

1. Protease inhibitors

1.1. Definition

Naturally occuring protease inhibitors (PI) which are protein in nature, are an important class of antinutritional factors (ANFs) in legume seeds (Liener and Kakade, 1980; Liener, 1989). They differ in specificity and stochiometry when bound to their substrates (Richardson, 1980;

Birk, 1989). Within this broad category of protease inhibitors, the main ones in legume seeds inhibit specifically trypsin and chymotrypsin, and some other serine proteases (Hathcock, 1991).

Protease inhibitors have been intensively studied in soybeans because of their wide use in human and animal nutrition. They constitute at least 6% of the proteins of soybeans (Ryan, 1973). Two major types of PI have been precisely identified in soybeans: the double-headed Bowman-Birk inhibitor with one binding site for trypsin and another for chymotrypsin (MW 8000 daltons); the single-headed Kunitz inhibitor with one binding site for trypsin only (MW 20000 daltons) (Brandon *et al.*, 1988). The Bowman-Birk inhibitor and the Kunitz inhibitor are proteins consisting respectively of 71 and 181 amino acids. The former contains seven disulfide bridges, compared to two in the latter.

1.2. Reactive site

The protease inhibitors react in a competitive manner with the enzymes and form a very stable complex ($K_{assoc.} = 10^8 - 10^{13}$ M^{-1}), thus completely removing the activity of the enzyme (Burns, 1987; Birk, 1989). To form such a complex, a section of 10 to 15 amino acids of the inhibitor enters into direct molecular contact with the active centre of the enzyme (Birk, 1989). A specific peptide bond of the inhibitor, recognized by the active site of the target enzyme, is hydrolysed. This hydrolysis does not change the conformation of the inhibitor because the reactive site is held within disulfide bridges in contrast to the target substrates of the enzyme (Birk, 1989). The modified inhibitor forms a stable complex with the enzyme. The specificity towards trypsin or chymotrypsin is due to the type of one of the amino acids involved in the peptide bond of the inhibitor; lysine or arginine for trypsin and phenylalanine, tyrosine or tryptophan for chymotrypsin.

2. Nutritional effects of protease inhibitors

The protease inhibitors family is of particular interest for animal nutrition as their characteristics are directly related to digestive enzymes secreted by the pancreas. The nutritional consequences of dietary protease inhibitors have been reviewed by Liener and Kakade (1980), Gallaher and Schneeman (1986) and Huisman and Jansman (1991). After an overview of the main effects, the factors that could affect the response to dietary protease inhibitors are discussed.

2.1. Hypothesis on mechanisms

Feeding soybean protease inhibitors to animal results generally in a low growth rate, along with a low nitrogen ileal or fecal apparent digestibility and a high deficiency of sulfur-containing amino acids (Nitsan and Liener, 1976b; Hasdai and Liener, 1983; Khorasani *et al.*, 1989).

It is generally accepted that the low nitrogen apparent digestibility can be due to:

- a high amount of endogenous nitrogen in the ileal digesta because of an increased secretion of enzyme from the exocrine pancreas or a reduced proteolysis and absorption of the endogenous protein in the small intestine (Nitsan and Liener, 1976*a*; Gallaher and Schneeman, 1986),
- a high amount of dietary proteins escaping digestion because of pancreatic proteases inhibition.

Increased secretion of pancreatic enzymes has been observed in several studies using rats, mice and chicken fed diets including protease inhibitors (Gertler and Nitsan, 1970; Laporte and Trémolières, 1973; Roy and Schneeman, 1981; Temler *et al.*, 1984). Upon entering the intestinal lumen, the protease inhibitors would inactivate pancreatic trypsin or chymotrypsin, thereby preventing the normal feedback regulation and stimulating pancreatic secretion via CCK hormone (Liener and Kakade, 1980). This increased secretion may also cause a pancreatic enlargement mediated by the CCK stimulating effect (Liener and Kakade, 1980).

Growth depression has been observed on rats fed diets containing free amino acids which do not require intestinal digestion, when protease inhibitors preparations were incorporated in the meal (Liener and Kakade, 1980). Therefore the antinutritive effects of protease inhibitors cannot be explained only by the inhibition of protease activity in the gut.

The physiological response to ingestion of PI-containing diet is species dependent (Struthers *et al.*, 1983; Gallaher and Schneeman, 1986; Huisman *et al.*, 1990*a*, 1990*c*). Tables 1 and 2 review the effects of PI feeding on pancreatic tissue and enzyme activity in pancreatic secretions and in ileal chyme of piglets and calves. It is shown that pancreas weight is almost unaffected in these animal species. Total output of proteases in the pancretic secretions did not increase. Enzyme activities in the pancreas and in the intestinal chyme decreased or remained at the same levels upon PI feeding. Increases of enzyme activities were rarely observed. Several explanations could be expressed. Protease inhibitors would bind trypsin and prevent the complete activation of chymotrypsinogen as observed by Lyman *et al.* (1974) in rats. Less oligo-peptides would then be released, and available to suppress the negative feedback mechanism and stimulate the pancreas. More dietary proteins would then escape digestion, leading to a poor digestibility and a growth inhibition. It could also be that, although part of

the proteases are inhibited, there would still be enough active enzymes to hydrolyse the dietary proteins. The differences could also be due to an inability of the pancreas to respond to the CCK stimulation in relation to the nutritional status of the animal (Gallaher and Schneeman, 1986). A low digestible diet could for instance lead to a lack of specific amino acids at a specific time, impairing pancreatic function.

There are numerous reports in literature about the effects of PI on pancreatic enzymes. However, their interpretation and comparison are difficult because several factors affect the response of the pancreas to PI, such as quality and quantity of the protein, or total sulfur amino acids content of the diet (Gumbmann and Friedman, 1987).

2.2. Factors of variation

a. *Protein sources in diets*

The protein sources of a diet containing PI are important with regard to the supply of nutrients to the animal.

Feeding mice with a 24% casein diet with or without a PI fraction led to the same growth rates (Roy and Schneeman, 1981). Green and Nasset (1983) measured in rats a lower pancreatic response to a casein diet than to a soy protein isolate diet devoid of PI, which is in agreement with Berger and Schneeman (1986). Likewise an unheated inhibitor-free soybean extract fed to rats did not have a much better nutritional value than raw soybean extract (Kakade *et al.*, 1973). The refractory nature of native soybean proteins to protease attack was believed to be responsible (Kakade *et al.*, 1973). Two aspects can be discussed for the contribution of protein quality towards variable results.

First, the proportions of endogenous proteins in the ileal chyme can be enhanced irrespective of PI. It can be due to a slower turnover of the endogenous proteins as intraluminal pancreatic proteases can accumulate when the dietary proteins are poorly digestible (Percival and Schneeman, 1979). Also, the binding of intraluminal proteases to dietary proteins could reverse protease-induced inhibition of CCK release, resulting in an increased pancreatic secretion (Kakade *et al.*, 1973; Green and Nasset, 1983). This mechanism could explain why in pigs, pair-feeding of one meal resulted in an increased protease content of the pancreas when the PI were given in the form of raw soybean meal, and in an unchanged content when they were given in an isolated form added to heated soybean meal (Yen *et al.*, 1977) (Table 1).

The second aspect concerns the amino acid availability. If the dietary proteins are poorly digestible or lack essential amino acids, the pool of amino acids available for protein metabolism may be unbalanced in the essential ones. In such conditions, the pancreas, when

stimulated via the feedback mechanism, may not be able to respond by increasing protease synthesis and secretion into the intestine (Myer *et al.*, 1982). In addition, the inadequacy of amino acids availability is enhanced by the fact that PI, the only proteins rich in cystine in peas for example, are not digested because they form complexes with proteins.

b. *Protein content of diets*

Protein quantity in the diet can also be a factor for variable results. The pancreas of rats fed PI enriched diets was unable to respond to the CCK stimulation induced by the PI when the casein content of the diets was as low as 5%. Plasma CCK stayed high even after 7 days, pancreas weight did not increased and trypsin activity in the proximal small intestine stayed low, for the 5% casein+PI diet. With the diets at 10% and 20% casein+PI, plasma CCK return to control level after 7 days, pancreas weight increased and trypsin activity in the intestinal chyme was higher than with the 5% casein diet (Green *et al.*, 1986).

c. *Feeding strategy*

The feeding strategy is also an important factor in the response of the pancreas to PI (Yen *et al.*, 1977) (Table 1). Compared to force-feeding of one meal, the rate of consumption was higher for the pair-fed meal. The consequences were probably a higher passage rate of PI in the duodenum and probably a higher stimulation of trypsin and chymotrypsin secretion from the pancreas, as measured in this trial (Yen *et al.*, 1977). Likewise, the negative effects of raw soybean meal were much more pronounced in the meal-fed rats than those fed *ad libitum* (Nitsan *et al.*, 1983; Nitsan and Nir, 1986).

d. *Duration*

The variation in length of time of PI exposure can also lead to variation in results. An *ad libitum* feeding period of 2 weeks did not affect pancreatic trypsin and chymotrypsin activities but a period of 6 weeks resulted in their decrease in the pancreas and in the intestinal chyme (Yen *et al.*, 1977) (Table 1). As for pair-feeding, it seems that in pigs, the pancreas can respond to a stimulation induced by dietary PI fed once. However, it would not be able to respond anymore when such a PI diet is fed for one week, as can be deduced from enzyme activities and granule density in the pancreas (Table 1).

The time effects may also vary with the protein sources as discussed previously. If the PI diets are based on low digestible proteins, the pancreas may lack at a certain time specific amino acids to synthesise extra enzymes if required.

e. *Other factors*

It has been observed that rats housed at 30°C fed raw soybeans as compared to heated soybeans had a lower growth rate than rats housed at 10°C (Borchers, 1965). Therefore at low environmental temperature, the metabolic adaptations in rats seem to avoid the common negative effects of raw soybeans, thus of PI as they have been shown from calculations to be responsible for at least 40% of the negative effects of soybeans (Nitsan and Liener, 1976*a*).

The age of the animal can also influence the effects of PI, as the protein requirements decrease with age (Saxena *et al.*, 1963).

Animal sex would be also a variation factor since young gilts seem to be more sensitive than young barrows to soybean PI, in relation to higher amino acids requirements (Yen *et al.*, 1974).

There are suggestions that antibiotics can decrease the negative effects of PI, especially in case of amino acids deficiency, by increasing the absorption from the lower intestine of some amino acids that are normally degraded by bacteria (Coates *et al.*, 1970).

Table 2. *Variations (↑: increase, ↓: decrease, ↔: unchanged) of protease activities in pancreatic secretions collected from pigs (35kg) or calves (3-8d old)*

	juice	protein		TA		SA		
	volume	mg/ml	output	T	CT	T	CT	References
PIGS								
26% RSBM *vs.* HSBM (8 d)	↑	↓ns	↔			↑	↔	Corring *et al.*, 1986
12% CP RSBM vs. HSBM (7d)	↑		↔	↑ns	↔	↔	↔	Zebrowska *et al.*, 1985
30% RSBM .vs. toasted SBM 1 meal	↑		↑			↑	↑	Souffrant *et al.*, 1985
CALVES								
15%cooked soyflour *vs.* milk diet	↓	↓		↓	↓	↓	↓	Gorrill *et al.*, 1967
35% soy isolate *vs.* milk diet (13d)	↔	↔		↓				Khorasani *et al.*, 1989
soyflour+milk *vs.* milk diet (6d)	↔			↔	↓			Ternouth *et al.*, 1975
fish protein conc.+milk *vs.* milk diet (6d)	↔			↔	↓			

TA: total activity, SA: specific activity (/mg protein), T: trypsin, CT: chymotrypsin,
RSBM: raw soybean meal, HSBM: heated soybean meal, conc.: concentrate, d: day

Table 1. *Variations (↑: increase, ↓: decrease, ↔: unchanged) of protease activities in pancreatic tissue and ileal chyme of piglets and calves*

		Pancreas						Intestinal chyme		
	Feeding state	weight	TA T	TA CT	SA T	SA CT	granule dens.	T	CT	References
PIGLETS (10-15 kg)						*Raw protein sources*				
30% pea *vs.* casein diet		↔								Huisman *et al.*, 1990a
25% S-pea + fish + milk vs. milk diet (12 days)	3h p.p.	↑				↔		↑ns	↑ns	Bertrand *et al.*, 1988
45% raw *vs.* extruded pea (S or W-pea) 2w., pair-feeding	24h p.p.	↔	↔	↓	↔	↔				Bengala Freire *et al.*, 1991
8% RSB + fish + milk	3h p.p.									Bertrand *et al.*, 1988
vs. 25% HSBM (12 days)		↔				↓ns		↔	↔	
vs. milk diet (12 days)		↑				↑ns		↔	↔	
RSBM *vs.* HSBM										Yen *et al.*, 1977
pair-feeding:										
-1m. after 7m. HSBM	2h p.p.	↔	↑	↑ns	↑	↑ns	↑	↓	↓	
-1w. after 1w. HSBM	2h p.p.	↔	↓	↓	↓	↓	↓	↓	↓	
-2w. after 1w. HSBM	2h p.p.	↔	↓	↓	↓	↓	↓	↓	↓	
force-feeding:										
-1m. after 7m. HSBM	2h p.p.	↔	↓ns	↓ns	↓ns	↓ns	↓	↓	↓	
-5m. after 7m. HSBM	2h p.p.	↔	↓	↓	↓	↓	↓	↓	↓	
ad libitum:										
-2w. RSBM	2h p.p.	↔	↔	↔	↔	↔	↔	↓	↔	
-6w. RSBM	2h p.p.	↔	↓	↓	↓	↓	↓	↓	↓	
20% RSBM vs. HSBM (4w.)	12h p.p.	↔			↓	↓				Struthers *et al.*, 1983
					Addition of SBTI					
HSBM + SBTI vs. HSBM										Yen *et al.*, 1977
pair-feeding:										
-1m. after 7m. HSBM	2h p.p.	↔	↔	↔	↔	↔	↔	↓	↓	
force-feeding:										
-1m. after 7m. HSBM	2h p.p.	↔	↔	↔	↔	↔	↔	↓	↓	
-5m. after 7m. HSBM	2h p.p.	↔	↓	↓	↓	↓	↓	↓	↓	
CALVES						*Raw protein sources*				
soja concentrate vs. milk diet (9w.)	12h p.p.		↓	↓	↓	↓	↓			Guilloteau *et al.*, 1986
Soyflour vs. soy concentrate		↔	↔	↔				↓	↓	Gorill and Thomas, 1967
Soyflour *vs.* milk diet			↓	↓				↓	↓	

TA: total activity, SA: specific activity (/mg protein or chyme), T: trypsin, CT: chymotrypsin, dens.: density, S: spring pea, W: winter pea,
RSB: raw soybean, RSBM: raw soybean meal, HSBM: heated soybean meal, SBTI: soybean trypsin inhibitors, conc.: concentrate m.: meal, w.: week, p.p.: post-prandial

PEA PROTEIN

Pea contains 20 - 25% crude protein. The water soluble proteins (80% of the total proteins) are composed mainly of albumins (20 - 25%) and globulins (55 - 65%). A high variability of the proportions of albumin and globulin has been observed, along with a strong negative correlation between the proportions of these two fractions (Guéguen and Barbot, 1988). The ratio "albumin / globulin" can vary between 0.19 and 1.14 within the same cultivar (Guéguen and Barbot, 1988).

Pea proteins are rich in lysine (7%) but deficient in sulfur amino acids (1.4% cysteine, 1.0% methionine, on total AA) with tryptophan (0.9%) as the third limiting amino acid. This deficiency comes from the globulin fraction as pea albumins can contain as much as 68% of the total protein sulfur (Schroeder, 1984) (Table 3).

Table 3. *Content of cystine, methionine and tryptophan in globulin and albumin from peas (From Guéguen, 1990)*

% of total AA	CYS	MET	TRP
globulin	0.80	0.70	0.67
albumin	3.15	1.34	1.47

1. Globulins

The globulin fraction contains the major seed storage proteins **legumin** and **vicilin**. They have respectively a molecular weight of 380000 daltons and 150000 daltons, a sedimentation value of 11 - 12S and 7 - 8S, and an isoelectric point of 4.8 and 5.5 (Guéguen, 1990). An important variation of the relative proportions of vicilin and legumin is observed; the ratios can vary from 0.5 to 5 and the legumin can represent 33 to 55% of the globulins (Guéguen and Baniel, 1990).

1.1. Legumin

Legumin is an oligomeric molecule made of 6 α-polypeptides (MW 40000) and 6 ß-polypeptides (MW 20000); one α-chain being linked to one ß-chain by disulphide bonds. The disulphide bonds and the high hydrophobicity of the (αß) subunits lead to a closely-packed and rigid structure. The subunits of pea legumin are very heterogeneous; their molecular weight ranges from 44000 daltons to 66000 daltons (Guéguen, 1990).

Legumin is higher in sulfur amino acids and tryptophan, but lower in isoleucine, lysine and phenylalanine compared to vicilin (Savage and Deo, 1989).
The homology between the different legumins is high not only within pea seeds but also between different legume seeds. The homology between pea legumin and cereal globulins exist also (Guéguen, 1990; Guéguen and Baniel, 1990).

1.2 Vicilin

The basic structure of vicilin, the other major globulin, would be a trimer of 50000 daltons subunits but with a high heterogeneity of subunits (MW 13000 to 50000). The subunits are not linked by disulphide bridges like legumin but probably by non-covalent forces because of the absence of cysteine in vicilin (Guéguen, 1990). For this reason, vicilin would be less heat-stable than legumin.

2. Albumins

The albumins include most of the enzymic and metabolic proteins. They contain two major polypeptides (MW $\approx$ 8000 and $\approx$ 22000) which together make up 34% of the total albumin fraction (Schroeder, 1984), and many other polypeptides.
Protease inhibitors belong to this category. In contrast to soybeans, little is known about the biochemistry of pea protease inhibitors. By electrophoresis, 8 bands of inhibitors were detected and after separation were found to have a molecular weight of about 16500 daltons (Tomé *et al.*, 1981). The activity of the pea protease inhibitors is in the range of 2 to 12 TUI/mg DM for spring to winter peas (Valdebouze and Gaborit, 1985). Peas have also a chymotrypsin inhibitor activity: 3.3 CUI/mg DM (Griffiths, 1984).
The albumin fraction contains another category of proteins named lectins for their specific ability to bind monosaccharides. According to Kornfeld *et al.* (1981) fucose would be an important determinant, but not the sole, in the carbohydrate-binding specificity of pea lectins. Lectins consist of two identical subunits, each containing an α- and a ß-polypeptide chain (5100-5800 daltons and 17500 daltons) (Meehan *et al.*, 1982). The lectin content in peas, determined by ELISA technique, is 3 to 5mg/g pea flour (Bertrand *et al.*, 1985; Huisman *et al.*, 1990*b*).
In the digestive tract, lectins exhibit antinutritional effects varying according to the genotype and the animal species. Pea lectins can bind to intestinal disaccharidases and proteases in the rat (Jindal *et al.*, 1982) but not to lipase (Aubry and Boucrot, 1986). The results could be then a reduced absorption of dietary nutrients among other effects. However, no adsorption of pea

lectins on intestinal mucosa has been observed in rats (Aubry and Boucrot, 1986) and in piglets (Bertrand *et al.*, 1988).

CONCLUSION

The present review is aimed at deriving a better understanding of the physiological role of the pancreas in protein digestion, especially when protease inhibitors are included in diet for pigs. The role of the pancreas in protein digestion is essential but obviously complex. Several factors in the process of dietary protein digestion in the gastrointestinal tract can condition the response of the exocrine pancreas. These include:

- protein sources and content in the diet,
- extend of gastric protein hydrolysis,
- activity of trypsin upon which depends the activation of the other zymogens,
- hormonal regulators.

The intake of dietary PI can effect the way in which these different steps are effective. The exocrine pancreas would be stimulated, via a gastrointestinal hormone, to secrete more proteases, when part of the secreted proteases are inhibited by PI in the intestinal lumen. This mechanism has been formulated from numerous observations on rats fed soybean trypsin inhibitors (Liener, 1976; Struthers *et al.*, 1983*b*; Gumbmann *et al.*, 1986). But from the literature, there is obviously an animal specie effect concerning the response to PI (Liener and Kakade, 1980; Gallaher and Schneeman, 1986; Huisman *et al.*, 1990*c*), as well as an interaction with the dietary components as described previously.

The poor nitrogen apparent digestibility of pea diet for young pigs could partly be due to the inhibition of pancreatic proteases by the PI in the small intestine. Kakade *et al.* (1973) have shown that PI accounted for 40% of the negative effect of raw soybeans fed to rats. For pigs, it is not clear if the poor digestibility is due to high amounts of exogenous or(and) endogenous proteins, as it is not known precisely if the pancreas of pigs secrete more proteases when pea PI fed. The interaction between pancreatic protease secretion and protease inhibition in the gastrointestinal tract should therefore be evaluated.

The production of pea ANF concentrates particularly rich in PI but not completely free of lectins, at INRA-Nantes (France) according to Guéguen (1983), can allow for the study of pea PI effects on pancreas of pigs. Protein quality in terms of amino acids availability is often used as one explanation when lower protease activities are found (Green *et al.*, 1986). The addition of ANF concentrate to different types of protein sources could highlight the importance of dietary amino acids availability. Measurements of levels of pancreatic proteases for diets containing ANF concentrates could perhaps be a method in determining threshold levels of PI for a specific animal species.

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Chapter III

NITROGEN ILEAL DIGESTIBILITY OF DIETS BASED ON RAW PEAS OR ISOLATED PEA PROTEINS FED TO PIGLET

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NITROGEN ILEAL DIGESTIBILITY OF DIETS BASED ON RAW PEAS OR ISOLATED PEA PROTEINS FED TO PIGLET

Four ileal digestibility experiments were designed to investigate digestibility of raw pea and two of its components - an isolate of its proteins and a concentrate of its proteinaceous antinutritional factors (ANFs). Pea protein isolates (PP), devoid of pea carbohydrates and of low trypsin inhibitor activities (TIA: < 2 mg inhibited trypsin / g), were produced from two varieties of peas - winter pea Frijaune (FR, TIA = 5.5), and spring pea Finale (FI, TIA = 1.2). One concentrate of ANFs was also produced from both pea varieties (batch a, TIA = 50). An additional batch (batch b, TIA = 163), more concentrated in trypsin inhibitors than batch a, was produced from FR pea.

Eleven semi-synthetic diets containing the PP isolates, the pea ANF concentrates or the raw peas (RP) were fed to piglets (10 - 15kg live weight) fitted with PVTC cannula. The apparent ileal N digestibility coefficients were 69.1 and 69.5 for the FI and FR raw pea diets, respectively. The N digestibility coefficients for the Finale PP diet and the Frijaune PP diet were 83.7 and 85.4 respectively. The ANF concentrate (batch a) incorporated at 3% level, reduced the N digestibility coefficient of the Finale PP diet to 79%. Addition of 0.6% of the batch b ANF concentrate reduced the N digestibility coefficient when the diet was based on raw pea (- 3 units) and not when based on PP.

Ileal digestibility : Peas: Antinutritional factors: Trypsin inhibitors: Piglets

INTRODUCTION

White-flowered peas (*Pisum sativum*) are considered to be valuable dietary protein source for both animals and humans. Nevertheless, growth performance of piglets have been shown to be depressed when the diets, balanced in essential amino acids, contained 40% spring peas (Jondreville *et al.*, 1992), or 15, 30 or 45% spring peas (Bengala Freire *et al.*, 1989). However, in other experiments, incorporation of 30% spring peas in piglet diets balanced in

amino acids did not affect growth performance (Gatel *et al.*, 1989; Grosjean *et al.*, 1991). Among pea chemical constituents, several may be responsible for the reduced growth performance. Peas contain protease inhibitors (Valdebouze *et al.*, 1980; Griffiths, 1984) and lectins (Meehan *et al.*, 1982), both being proteinaceous antinutritional factors (ANFs). Pea protein digestibility could be affected by protease inhibitors (Leterme *et al.*, 1990). Starch has also been sometimes incriminated as a possible negative factor (Longstaff and McNab, 1987; Bengala Freire *et al.*, 1988) in relation to its low susceptibility to hydrolysis (Colonna and Mercier, 1979; Colonna *et al.*, 1992).

The α-galactosides, soluble carbohydrates present in peas at a level of about 5% (Quemener and Mercier, 1980), may also be responsible. As the digestive tract is not equipped with adequate enzymes to hydrolyse α-galactosides, their presence would lead to bacterial fermentation in the hindgut, and possibly flatulence and diarrhoea (Cristofaro *et al.*, 1974; Saini, 1989)

The objective of this study was to understand the digestion of raw smooth seed peas by piglets, through digestibility experiments with diets containing pea protein isolate low in ANFs and with diets enriched in pea ANF concentrates. In a separate experiment, it was found that isolated pea carbohydrates did not affect the nitrogen apparent ileal digestibility of a pea protein isolate diet (Huisman *et al.*, 1990).

MATERIALS AND METHODS

Four experiments with piglets were carried out to measure the apparent ileal digestibility of diets containing high levels of raw peas (RP) or pea protein isolates (PP) of the spring variety Finale (experiment 1) and the winter variety Frijaune (experiment 2). The influence of an addition of pea ANF concentrate to the Finale PP diet on digestibility was measured in experiment 3. The interaction between ANFs and pea protein sources (PP or RP) on digestibility was measured in experiment 4.

1. Origin of the pea protein sources

The spring peas Finale (FI) and the winter peas Frijaune (FR) used in experiments 1 & 2 respectively, were harvested in France in 1988. In experiment 4, spring peas Solara (SO) harvested in France in 1990, were used. The peas were ground through a 2.5mm screen. Their compositions are reported in table 1.

Two protein concentrates (one from Finale, one from Frijaune) were prepared. They consisted in the light and fine fraction of particles separated from the pea meal by an air-classifier set at

a cut point of approximately 15μm, according to van der Poel *et al.* (1989). They were used in the diets in order to enhance the native pea protein content and to avoid high level of pea carbohydrates as with inclusion of 60% raw peas.

Three pea protein isolates (PP) of different origins and processes were used. Two protein isolates, one from Frijaune and one from Finale, were prepared at INRA research centre at Nantes (France) by acid precipitation at pH 4.5, according to Guéguen (1983). The other protein isolate, produced by ultrafiltration, was bought from Nutrio in Denmark (P-Pro 2000 pea protein isolate).

Three pea ANF concentrates were produced by diafiltration and ultrafiltration, at INRA-Nantes, from the whey fractions left from the preparation of the pea protein isolate produced by precipitation (Guéguen and Bérot, unpublished results). Two ANF concentrates were prepared in 1988 (one from Finale, one from Frijaune), and mixed together as 1:1 (batch a). A second ANF concentrate from Frijaune (batch b) was prepared in 1990 using the same Frijaune whey fraction as used in 1988. The compositions of both batches are reported in table 1. The trypsin inhibitor activity was three times higher in batch *b* than in batch *a*.

Table 1. *Chemical composition (g/100 g product) of pea protein sources*

Product...	Raw pea			PP			ANF concentrate	
Origin...	FI	FR	SO	FI	FR	Com.	batch a	batch b
DM	87.1	87.1	nd	96.5	95.9	94.4	nd	nd
CP	23.7	21.9	20.0	93.1	88.3	87.8	67	70
TIA[a]	1.2	5.5	1.3	0.6	1.6	1.0	50	163
Lectins[b]	3.5	3.6	2.8	1.4	1.6	6.6	102	52

FI, FR, SO: Finale, Frijaune and Solara varieties, com: commercial PP, batch a: 50% from FI + 50% from FR, batch b: 100% from FR, nd: not determined

[a] TIA, trypsin inhibitor activity; in mg inhibited trypsin/g product.

[b] Lectins: in mg/g product.

2. Diets

The diet compositions are given in Table 2 (experiments 1, 2, 3) and in Table 3 (experiment 4).

Experiment 1: Three diets were formulated based on:
- fish meal and casein: control diet (*C1*),
- raw pea and air-classified pea protein from spring pea Finale (*RP1*),
- pea protein isolate from spring pea Finale (*PP1*).

Experiment 2: Three diets were formulated based on:
- fish meal and casein: control diet (*C2*),
- raw pea and air-classified pea protein from winter pea Frijaune (*RP2*),
- pea protein isolate from winter pea Frijaune (*PP2*).

Table 2. *Composition (g/100g feed) of the diets fed to piglets in experiments 1, 2, 3*

Experiment...	1,2	1	1, 3	2		3
Diet ...	*C1, C2*	*RP1*	*PP1, PP3*	*RP2*	*PP2*	*PP3+*
Fish meal	6.9	-	-	-	-	-
Casein	12.5	-	-	-	-	-
Finale raw pea	-	25.0	-	-	-	-
Finale air-classified pea protein	-	18.6	-	-	-	-
Frijaune raw pea	-	-	-	25.0	-	-
Frijaune air-classified pea protein	-	-	-	17.8	-	-
INRA pea protein isolate (Frijaune)	-	-	-	-	17.9	-
INRA pea protein isolate (Finale)	-	-	18.4	-	-	16.4
Pea ANF concentrate (batch a)	-	-	-	-	-	2.9
Maize starch	51.8	30.0	52.2	30.7	52.7	51.3
Cellulose (Arbocell B 800)	5.0	3.0	4.8	3.0	4.8	4.8
$CaCO_3$	1.2	1.5	1.5	1.5	1.5	1.5
$CaHPO_4$	2.0	2.0	2.4	2.0	2.4	2.4
$KHCO_3$	1.7	.5	1.1	.5	1.1	1.1
DL-methionine	.06	.28	.37	.29	.38	.37
L-threonine	-	.06	.16	.14	.14	.16
L-tryptophan	-	.06	.06	.06	.06	.06
Chemical composition: analysed (% DM)						
Net Energy (Mcal)[a]	.27	.27	.26	.27	.27	.27
Crude protein	19.0	18.8	19.3	18.3	18.4	19.5
Crude fibre[a]	5.6	5.7	5.5	5.7	5.5	5.6
Methionine+Cystine	.70	.77	.73	.69	.71	.72
Lysine	1.32	1.06	1.17	1.05	1.14	1.15
Tryptophan	.21	.20	.21	.20	.21	.21
TIA[b]	nd	.7	.1	1.9	.4	1.2
Lectins[c]	nd	2.3	.4	1.9	.5	2.7

RP: raw pea; *PP*: pea protein isolate.

All diets contained in addition (g/100g feed): dextrose (15.0), sunflower oil (2.0), vitamin/mineral mix (1.0), NaCl (.5), $NaHCO_3$ (.4), Chromic oxide (.1)

The vitamin/mineral mix provided (mg/kg feed): Retinol 2.7, cholecalciferol 0.045, DL-α-Tocopherol 40, menadione 3, riboflavine 5, nicotinic acid 30, D-pantothenic acid 15, choline chloride 120, cyanocobalamin 0.04, ascorbic acid 50, $CuSO_4.5H_2O$ 20, $ZnSO_4.H_2O$ 200, MnO 70, $FeSO_4.7H_2O$ 400, $CoSO_4.7H_2O$ 2.5, $Na_2SeO_3.5H_2O$ 0.2, KI 0.5.

[a]: calculated values

[b] TIA, trypsin inhibitor activity; in mg inhibited trypsin/g feed.

[c] Lectins: in mg/g feed.

Experiment 3: Two diets were formulated based on:
- pea protein isolate from spring pea Finale (*PP3, =PP1*).
- *PP3* + 3% ANF concentrate from Finale and Frijaune (1:1, batch *a*) ($PP3^{+}$).

The ANF concentrate could not be added at a higher level than 3% because of limited quantities available, although the aim was to reach the same trypsin inhibitor activity as in the *RP2* diet.

Experiment 4: Five diets were formulated based on:
- commercial pea protein isolate (*PP4*).
- *PP4* + 0.6% ANF concentrate from Frijaune (batch *b*) ($PP4^{++}$).
- Solara raw pea and commercial pea protein isolate (*RPP4*),
- *RPP4* + 0.4% ANF concentrate from Frijaune ($RPP4^{+}$),
- *RPP4* + 0.6% ANF concentrate from Frijaune ($RPP4^{++}$).

Two incorporation levels of ANF concentrate were used for the *RPP4* diets, in order to balance with the $PP4^{++}$ diet on TI activity or on the amount of ANF concentrate.

Table 3. *Composition (g/100g feed) of the diets fed to piglets in experiment 4*

Diets...	*PP4*	$PP4^{++}$	*RPP4*	$RPP4^{+}$	$RPP4^{++}$
Solara raw pea	-	-	30.0	30.0	30.0
Commercial PP	15.0	15.0	9.3	9.3	9.3
INRA PP (50%Fi+50%Fr)	4.0	4.0	2.4	2.4	2.4
Pea ANF concentrate(batch b)	-	0.6	-	0.4	0.6
Maize starch	51.4	50.8	29.2	28.6	28.8
$KHCO_3$	1.1	1.1	.6	.6	.6
DL-methionine	.38	.38	.35	.35	.35
L-threonine	.15	.15	.13	.13	.13
L-tryptophan	.07	.07	.07	.07	.07
Chemical composition: calculated(% DM)					
Net Energy (Mcal)	.27	.26	.26	.26	.26
Crude protein (analysed)	18.6	19.2	18.1	18.4	18.6
Crude fibre	5.7	5.7	7.8	7.8	7.8
Methionine+Cystine	.75	.74	.78	.78	.78
Lysine	1.33	1.32	1.38	1.38	1.39
Tryptophan	.23	.23	.25	.25	.25
TIA[a]	.1	.8	.4	.9	1.1
Lectins[b]	1.5	1.9	1.9	2.5	3.4

RP: raw pea; *PP*: pea protein isolate. *Fi, Fr*: spring pea Finale and winter pea Frijaune.
All diets contained in addition (g/100g feed): dextrose (15.0), sunflower oil (2.0), cellulose-Arbocell B 800 (5.0), vitamin/mineral mix (1.0), NaCl (.5), $CaCO_3$ (1.6), $CaHPO_4$ (2.4), $NaHCO_3$ (.4), Chromic oxide (.1)
[a] measured TIA, trypsin inhibitor activity; in mg inhibited trypsin/g feed.
[b] measured lectin content: in mg/g feed.

The diets were semi-purified diets and contained 16% to 18% crude protein exclusively from pea proteins except for the control diets. They were balanced in net energy according to CVB tables (1988) and in amino acids using synthetic amino acids. Purified cellulose was added to balance the crude fibre content.

Chromic oxide was included at a level of 0.1% in the diets as a digestibility marker.

The feed was pelleted (2.5 mm ∅) without steam, at a maximum temperature of 50 to 55°C. The trypsin inhibitor activity (TIA) and the lectin content of the diets are given in Table 2 (experiments 1, 2, 3) and in Table 3 (experiment 4).

The TIA of the PP diets (PP1, PP2, PP3, PP4) were low as planned (<0.4 mg trypsin inhibited/g feed). The TIA of the raw pea diets (RP1, RP2, RPP4) ranked from 0.4 to 1.5 mg, depending on the pea variety. The TIA of the diets enriched in ANFs ($PP3^+$, $PP4^{++}$, $RPP4^+$, $RPP4^{++}$) ranked from 0.8 to 1.2 mg. The levels of incorporation of the ANF concentrates ranged from 0.4 to 2.9% depending on the batch of ANF concentrates, as batch "b" had a TIA three times higher than batch "a".

As the commercial PP contains more lectins than the INRA PP, the PP4 diet had a higher lectin content than the PP1, PP2, and PP3 diets (1.5 mg *vs.* 0.5 mg, resp.). The RP diets and the ANF-concentrate enriched diets contained from 1.9 to 3.4 mg lectins/g feed.

3. Animal procedures

Piglets (74 total, 5 or 6 animals per treatment) of the crossbred Dutch Landrace x Yorkshire were obtained from one breeding farm and were allocated to the diets according to live weight and litter. The mean live weights of the animals per group were similar. Piglets from the same litter were allocated to different groups. All diets within an experiment were tested in one trial.

The piglets were housed individually in metabolism cages designed for ileum cannulated piglets. After one week of cage adaptation, at an age of 5 weeks and a live weight of 9 kg, they were surgically fitted with a post-valve T caecum cannula (PVTC cannula) at the terminal end of the ileum according to van Leeuwen *et al.* (1990), and allowed to recover for up to two weeks.

After the recovery period from the cannulation, the piglets were adapted to the experimental feed during 10 days in experiments 1 & 4. In experiments 2 & 3, 7 days feed adaptation were allowed as not enough feed could be made for a 10 day adaptation.

The pellets were fed as dry pellets twice daily (08.00 and 20.00 hours) at a restricted level of feed intake (2.6 times the maintenance energy requirement, ARC, 1981). Water was available *ad libitum* through a nipple drinker.

The ileal chyme were collected in plastic bags, 12 hours per day (08.00 - 20.00 hours) during 5 consecutive days (van Leeuwen *et al.*, 1990). Collecting bags were changed every hour and weighed before being immediately stored at -20°C. At the end of the collection period, the animals weighed 15 to 17kg.

4. Chemical analysis

Prior to chemical analysis, the chymes collected per animal were pooled, homogenized, sub-sampled and freeze-dried. The feed and digesta were ground through a 1-mm mesh screen.
The dry matter content was determined by drying the samples to constant weight at 105°C. Nitrogen was analysed according to AOAC (1980). Trypsin inhibitor activity was determined using the Kakade method as modified by van Oort *et al.* (1989). Lectin content was measured according to an ELISA technique (Hamer *et al.*, 1989). Chromic oxide content was determined following hydrolysis in a mixture of perchloric and nitric acids and measuring the Cr^{6+} by flame atomic absorption spectrophotometry.

5. Calculations and statistical procedures

Digestibility coefficients (DC) were calculated from the nutrient concentrations in the diets and in digesta samples. The amounts of collected digesta were corrected to the expected value using the chrome (Cr) recovery factor.
The following equations were used:

(1) $$\text{Cr recovery factor} = \frac{\%CR_f \times F - \%CR_c \times C}{\%CR_f \times F}$$

where $\%CR_f$ = % of Cr in the feed
$\%CR_c$ = % of Cr in the wet chyme
F = amount of feed intake (g)
C = amount of chyme collected (g)

(2) $$C_{cor} = C / (\text{Cr recovery factor})$$

where C_{cor} = corrected amount of chyme(g)

(3) $$DC_X = \frac{\%X_f \times F - \%X_c \times C_{cor}}{\%X_f \times F}$$

where $\%X_f$ = % of nutrient X in the feed
$\%X_c$ = % of nutrient X in the wet chyme

When calculating the digestibility of nitrogen, corrections were made assuming the synthetic amino acids to be completely absorbed in the small intestine (Huisman *et al.*, 1986). The N apparent DC were calculated for each diet, and corresponded actually to the N apparent DC of the respective protein sources. The results are given as means with their standard errors.

A one-way analysis of variance was carried out separately for each experiment, using the SPSS/PC software package (Norusis, 1988). The following model was used:

(4) $$Y_{ij} = \mu + D_i + e_{ij}$$

where Y_i = dependent variable, μ = mean, D_i = diet effect and e_i = residual error between animals. For experiments 1 & 2: i = 1, 2, 3, for experiment 3: i = 1, 2 and for experiment 4: i = 1 .. 5.

The results of experiment 4 were also combined in a two-way analysis of variance according to the following model:

(5) $$Y_{ijk} = \mu + P_i + A_j + (PA)_{ij} + e_{ijk}$$

where Y_{ij} = dependent variable, μ = mean, P_i = protein source effect (i = 1, 2), A_j = effect of level of ANF concentrate (j= 1, 2, 3), $(PA)_{ij}$ = interaction between the protein source and the ANF concentrate level, and e_{ij} = residual error between animals.

The significance of differences between treatment means was tested using the Least Significance Difference test (Snedecor and Cochran, 1980). All statements of significance are based on a probability of less than 0.05.

Correlation and linear regression calculations were also performed (SPSS package). Two explaining variables were considered: TIA and lectin contents in the diets. Three dependent variables were taken into account: daily weight gain (DWG), N ileal DC, or dry matter ileal DC. For these variables, the means obtained for each treatment group were taken into account, without correction for the experiment.

RESULTS

The mean daily weight gains of the animals was 276g/d (SE: 5). No feed refusals were observed. The chrome recovery was high (mean: 98%, SE: 1.0) (Tables 4 & 5), indicating adequate cannulation technique for ileal chyme collection under these experimental conditions. The daily intakes of feed were approximately the same for the piglets within the experiments 1, 2 and 3. However, the piglets of experiment 4 had a lower intake of feed (475 vs. 544g/d) and of N (12.7 vs. 14.7g/d) than the piglets of experiments 1, 2 and 3, because they had a lower live weight (9kg *vs.* 12kg).

The results concerning the raw pea diets of experiments 1 & 2 are reported in table 4. The amounts of chyme collected at the end of the ileum were 2 or 3 times higher when the piglets were fed the RP diets than when fed casein+fish meal or PP diets. The mean DM contents of the digesta were lower when RP were fed than when casein+fish or PP were fed, but not significantly different (p=0.24 & 0.42, experiments 1 & 2 resp.). The mean apparent ileal DC

of N and DM were the lowest for the RP diets: 69% and 75% respectively. The DC of the N were not influenced by the variety of pea (RP1 and RP2).

Table 4. *Chyme production and apparent ileal digestibility of pea diets fed to piglets of 10kg in experiments 1 & 2: mean and (standard error)*

	Experiment 1			Experiment 2		
Diets...	*C1*	*RP1*	*PP1*	*C2*	*RP2*	*PP2*
Ileal chyme						
Cr recovery %	95.0	100.4	92.8	96.1	105.2	99.6
	(2.4)	(6.2)	(3.3)	(3.7)	(4.8)	(2.3)
g wet/12h	291[a]	741[b]	333[a]	240[a]	529[b]	298[a]
	(35)	(56)	(38)	(22)	(22)	(49)
% DM	11.3	9.3	11.0	13.3	11.9	12.0
	(.9)	(.8)	(1.0)	(.9)	(.5)	(1.1)
g dry/12h	31.6[a]	66.8[b]	34.9[a]	31.3[a]	62.6[b]	33.3[a]
	(1.0)	(2.7)	(1.6)	(1.5)	(4.2)	(2.6)
Apparent ileal digestibility coefficients (%)						
DM	85.8[a]	73.9[b]	84.2[a]	86.3[a]	76.8[b]	86.9[a]
	(.3)	(.3)	(.9)	(.7)	(.5)	(.4)
N	82.4[a]	69.1[b]	83.7[a]	83.1[a]	69.5[b]	85.4[a]
	(1.0)	(1.8)	(1.6)	(1.0)	(1.7)	(.9)

Full names of the diets are mentioned in tables 2 & 3. Values within experiment and line with different superscripts differ ($p < 0.05$).

For the PP diets, in all experiments (tables 4 & 5), the total chyme production over 5 days ranged from 230 to 330g per 12 hours, at 11-12% dry matter content, as for the control diets of experiments 1 & 2. The apparent ileal DC of the PP diets were high and similar to those of the control diets (Tables 4 & 5). The DC values of the control and PP diets were about 84% for N and 86% for DM. The N digestibility of the PP diets was not affected by the variety of pea (Table 4), or by the process applied to obtain the isolate (precipitation or ultrafiltration, Tables 4 & 5).

The *RPP4* diet of experiment 4 (Table 5), containing 30% raw pea and 12% PP, led to intermediate results: chyme production of 370g and apparent ileal DC of 81% for N and 79% for DM.

The addition of pea ANF concentrate *(ANF batch a)* to pea protein isolate in experiment 3 *(PP3+* diet) decreased the ileal DC of N and DM by 7.1 points and 2.7 points respectively (Table 5). The amounts of dry chyme were increased (+16%, $p < 0.05$) with ANF concentrate addition.

In experiment 4 (Table 5), the ANF concentrate (*ANF batch b*) added to pea protein isolate (*PP4*$^{++}$ diet) did not affect DM and N ileal DC. However, when this ANF concentrate was added to "30% RP + 12% PP", the N and DM apparent ileal DC of the diet were reduced. At the highest incorporation level (0.6%), this reduction was significant for DC N (-2.9 units) . The amounts of chyme were not affected by the addition of ANF concentrates.

Table 5. *Chyme production and ileal digestibility of pea diets fed to piglets of 10kg in experiments 3 & 4: mean and (standard error)*

	Experiment 3		Experiment 4				
Diets...	*PP3*	*PP3*$^{+}$	*PP4*	*PP4*$^{++}$	*RPP4*	*RPP4*$^{+}$	*RPP4*$^{++}$
Ileal chyme							
Cr recovery %	94.7	89.5	92.2	98.7	100.3	102.9	99.1
	(3.7)	(3.0)	(2.1)	(2.2)	(1.4)	(2.4)	(3.0)
g wet/12h	278	396	229^{a}	220^{a}	372^{b}	382^{b}	398^{b}
	(51)	(39)	(21)	(11)	(18)	(7)	(19)
% DM	12.3	9.4	12.3	13.4	12.1	12.3	12.0
	(1.6)	(.8)	(.8)	(.7)	(.8)	(.4)	(.4)
g dry/12h	31.2^{a}	36.2^{b}	27.3^{a}	29.1^{a}	44.3^{b}	46.7^{b}	47.6^{b}
	(.7)	(1.1)	(.8)	(.7)	(1.1)	(1.7)	(1.6)
Apparent ileal digestibility coefficients (%)							
DM	86.8^{a}	84.1^{b}	86.2^{a}	86.3^{a}	79.1^{b}	77.9^{c}	78.5^{bc}
	(.4)	(.3)	(.1)	(.1)	(.5)	(.3)	(.3)
N	86.0^{a}	78.9^{b}	84.7^{a}	84.2^{a}	80.8^{b}	79.4^{bc}	77.9^{c}
	(1.0)	(1.3)	(.7)	(.6)	(1.0)	(.5)	(.8)

Full names of the diets are mentioned in tables 2 & 3. Values within experiment and line with different superscripts differ ($p < 0.05$).

The coefficients of linear correlation calculated between "TIA or lectin content in the feed" and "DWG, DC_{DM} or DC_N" ranged between -0.35 (lectin & DWG) and -0.66 (TIA & DC_N) (Table 6). The correlation between lectin content and DC_N was -0.50. The regression analysis between the same variables showed a significant ($p < 0.05$) contribution of TIA or lectins to the individual parameters with R^2 coefficients ranging from 0.11 to 0.36.

Table 6. *Coefficients of correlation between trypsin inhibitor activity (TIA) or lectin content in the feed and daily weight gain (DWG), dry matter and nitrogen digestibility coefficients (DC) (experiments 1, 2 3 & 4)*

	DWG	DC_{DM}	DC_N
n	10	13	13
TIA	-0.49	-0.53	-0.66
Lectins	-0.33	-0.60	-0.50

$p < 0.05$ for all coefficients. n: number of group means taken into account

DISCUSSION

The main goal of the experiments was to investigate in piglets the digestibility of raw pea in relation to two of its components - an isolate of its proteins (free from carbohydrates and antinutritional factors) and a concentrate of its antinutritional factors. The digestibility coefficients of N have been measured as apparent values. This means that no distinction has been made between dietary and non-dietary N in the chyme. A low apparent ileal N digestibility could be due to a high amount of endogenous N or/and a high amount of undigested dietary protein.

The apparent ileal digestibility coefficients of the French commercial smooth peas were low and similar for both varieties (Table 4). However, the winter pea had a higher trypsin inhibitor activity than the spring pea. The digestibility of the Solara pea used in experiment 4 was also low (approximately 72%). Large differences in protein digestibility between varieties of peas (*Pisum sativum*) fed to pigs have been reported in the literature (Green, 1988; Buraczewska *et al.*, 1989; Gdala *et al.*, 1992). When French commercial varieties of *Pisum sativum* are distinguished according to their spring or winter genotypes, it appears that the N apparent fecal DC cover two classes of values - higher for the spring peas than for the winter peas - but with an overlap of the classes: 75 - 88% for winter pea and 82 - 89% for spring pea (Perez and Bourdon, 1992). With pigs weighing 40 to 60 kg, the apparent ileal N digestibility, using the end-to-end ileo-rectal anastomosis method, of spring and winter peas can range between 61% and 80% (Jondreville *et al.*, 1992). The DC measured in the present study are consistent with the literature data.

The results of Bengala Freire *et al.* (1988) indicate a higher apparent ileal protein digestibility of Finale pea than measured in this study (79% *vs.* 69%), with piglets of about the same age in both studies. This difference could be due to a batch effect as the agro-climatic growing conditions can influence pea composition (Bacon *et al.*, 1992; Daveby, 1992). This can also mean that, apart from errors in determinations, young animals may vary in their reactions to peas in relation, for example, to weaning procedures (Makkink *et al.*, 1992). Other reasons could be a different ileal digestibility technique (end-to-end ileo-rectal anastomosis *vs.* PVTC cannulation) or the higher crude protein content of the diets (16% in the present diets *vs.* 25% in the study of Bengala Freire *et al.* (1988)).

The apparent ileal N digestibility increased by 15 units when the protein source consisted of a precipitated pea protein isolate devoid of pea carbohydrates and of pea ANFs, instead of raw pea (experiments 1 and 2, Table 4). It should be pointed out that the wet process for producing the precipitated PP leads to a protein fraction which is not exactly identical to the fraction in raw peas. The soluble protein fraction of raw peas contains 70-80% storage

proteins called globulins, and 20-30% biologically active proteins such as enzymes, protease inhibitors and lectins, which are called albumins (Guéguen and Barbot, 1988). In the pea protein isolate, these proportions are changed to about 90% globulins and 10% albumins as the latter proteins do not precipitate at pH 4.5 (Guéguen and Bérot, unpublished results). If the albumins have a low digestibility, as suggested in a study with radiolabelled pea lectins that partly passed undamaged the small intestine of rats (Aubry and Boucrot, 1986), the proteins of PP isolate may therefore be more digestible than the raw pea proteins.
The commercial ultrafiltered PP, used in experiment 4, could have the same proportions of albumins and globulins as that in raw peas (Guéguen *et al.*, 1983), depending on the molecular weight cut size of the ultrafiltration membranes used in the industry. An electrophoresis examination of the two isolates used in the present investigations indicated that the ultrafiltered PP did not contain more albumins than the one produced by precipitation (Guéguen, 1992, unpublished results). It would mean that the low molecular weight proteins (approximately < 50000 daltons) passed through the ultrafiltration membranes and were eliminated in the permeate. This is in agreement with the fact that both precipitated and ultrafiltered PP have similar high N apparent ileal DC.

The two fractions removed from the pea seeds - carbohydrates and ANFs - may be considered as responsible for the differences in digestibility coefficients between raw peas and PP.

It has been shown by Darcy *et al.* (1981) that the nature of the starch associated with a protein source has an influence on apparent ileal digestibility of N. Pea carbohydrates were added in a purified form to a PP diet and it was found that total pea carbohydrates, including starch, α-galactosides and hulls, did not affect N apparent ileal digestibility although they decreased the DM digestibility by 3 units (Huisman *et al.*, 1990). The inclusion of purified pea carbohydrates increased the amounts of wet chyme produced (+25 to +45%). However these amounts (350 to 420 g wet chyme/12 h) were not as high as for the RP diets of the present experiments (1 & 2).
Another dietary factor that may affect N and DM apparent ileal DC is the accessibility to digestive enzymes of the cytoplasmic content in relation to the cell walls. Due to the process of isolation of the major pea fractions, the accessibility of the proteins or carbohydrates to enzymes may actually have been improved by modifications of the native structure of the cells and possibly, modifications of the bonds between the constituents. The study by Carré *et al.* (1991) using chickens suggested that non-accessibility to the cytoplasmic content of the pea cells was a main factor affecting protein and starch digestibility of raw peas by chickens. For piglets, it could also be an important factor.

Other dietary constituents that may affect the N digestibility of the raw pea are ANFs, especially the protease inhibitors. Increased secretion of pancreatic enzymes has been observed in several studies using rats, mice and chickens fed diets including protease inhibitors (Gertler and Nitsan, 1970; Laporte and Trémolières, 1973; Roy and Schneeman, 1981; Temler *et al.*, 1984). In these studies, the mode of action of protease inhibitors could be described as follows: after entering the small intestinal lumen, the protease inhibitors inactivate pancreatic trypsin or chymotrypsin, thereby preventing the normal feedback regulation and stimulating pancreatic secretion via CCK hormone (Liener and Kakade, 1980). The result is an increase of endogenous N in the chyme, and therefore a decreased apparent N digestibility.

The ANFs influence in peas was studied by adding pea ANF concentrates to a diet. The proteins of these concentrates (crude protein content of 70% approximately, Table 1) were mainly albumins, consisting partly of protease inhibitors and lectins, as the other proteins, the globulins, were precipitated during the isolation process of the PP.

Experiment 3 clearly demonstrated that the addition of 2.9% pea ANF concentrate (batch a) to a PP diet, leading to a feed TIA of 1.2, reduced the apparent ileal N digestibility (Table 5). This reduction amounted to 7 units, which was about half the difference in N digestibility between the PP diet and the RP diet (15 - 16 units, Table 4).

In experiment 4, the addition of 0.6% pea ANF concentrate (batch b), leading to a feed TIA of 0.8, did not change the digestibility of the PP diet. The TIA of the ANF enriched PP diet was lower in experiment 4 than the TIA in experiment 3 (0.8 *vs.* 1.2). If there is a feed TIA threshold level and if it is higher than 0.8 units, then no effect would be found in experiment 4. Another difference between experiment 3 and experiment 4, is the ANF concentrate itself. The concentrate used in experiment 4 had a higher TIA and was from one variety (Frijaune) instead of two (Frijaune and Finale) in experiment 3. As has been shown by Tomé *et al.* (1981), spring peas have more iso-inhibitors than winter peas. Therefore the ANF concentrates used in experiments 3 and 4, probably contained different amounts of iso-inhibitors. They may therefore have had different effects on nutrient digestibility. In experiment 4, the ANF concentrate was included at a lower percentage than in experiment 3: 0.6% *vs.* 2.9% because of its higher TIA content. Assuming that the albumin proteins of the ANF concentrate were not digested at all, the N ileal digestibility of a PP diet to which 2.9% ANF concentrate were added, would decrease by 7 units as measured in experiment 3. This hypothesis is in good agreement with the study of Grant *et al.* (1986) who found in rats, a low nutritional value of a soybean whey protein fraction soluble at pH 4.8, equivalent to the ANF concentrate used in this study. This low nutritional value could not be directly correlated with the TIA of the fraction.

Although a low digestibility of the ANF concentrate itself cannot be ruled out, experiment 4 shows that this is not the only factor influencing the effects of ANF concentrate observed on digestibility in experiment 3. Added at a level of 0.6%, the pea ANF concentrate reduced the digestibility when the diet was based on raw pea. No reduction was found when the diet was based on PP. The different effect according to the protein sources could be accounted for the protease inhibitors. Yen *et al.* (1977) showed that pair-feeding of one meal of TI in the form of raw soybean meal to pigs increased the protease content of the pancreas. But when isolated TI were added to heated soybean meal, the pancreas was not affected. Likewise, mice fed a 24% casein diet had the same DWG whether a TI fraction was added to the diet or not (Roy and Schneeman, 1981). Protease inhibitors could be partly responsible for the reduction of N digestibility coefficients.

The correlation coefficient between TIA and N digestibility data measured in the present studies was -0.66 (Table 6). In agreement with this result, Perez and Bourdon (1992) calculated a coefficient of - 0.70 between TIA and N digestibility coefficients in 10 batches of French white-flowered peas (winter and spring type). TIA would therefore be a parameter explaining 50% of the variation of pea N digestibility. Koehler *et al.* (1988) found also that reducing TI content of soybeans through genetic selection allowed to recover 50% of the depression in apparent ileal N digestibility of raw soybeans with pigs. However the wide variation of ileal N digestibility of 10 pea varieties observed Gdala *et al.* (1992) in pigs was independent of the pea TIA.

The correlation coefficients mean also that about 50% of the variation of pea N digestibility coefficients would be explained by other elements than protease inhibitors.

Pea lectins could also affect the digestibility. Their role could be linked to their own low digestibility as they are themselves albumin proteins (Aubry & Boucrot, 1986). On the other hand, they could be toxic if they bind to carbohydrate moiety of the glycoproteins of the small intestinal brush border membrane (Kik, 1991). Concerning pea lectins, the literature reports conflicting views. Purified pea lectins did not affect piglets and rats in any way (Grant *et al.*, 1983; Bertrand *et al.* 1988; Aubry and Boucrot, 1986). On the other hand, Jindal *et al.* (1982) observed that they damaged the gutwall of rats.

Antigenicity of pea proteins may be another factor affecting the nutritional value of raw peas. In a pilot experiment, it was observed that piglets, fed the RP2 diet described in the present study, formed circulatory antibodies against pea proteins (Le Guen *et al.*, 1991). Disorders in gut motility and therefore in digestion efficiency may appear when antigenic proteins are fed, like soybean proteins in calves (Sissons et al., 1989). Pea lectins, if they damage partly the gut wall, could amplify such disorders and favour the uptake of antigenic proteins from the intestinal lumen into the blood.

CONCLUSION

Four experiments using piglets fed diets containing pea proteins showed lower apparent ileal N digestibility coefficients when the proteins consisted of raw peas rather than pea protein isolates. The pea ANFs were shown to be partly responsible for this difference. It could not be specified from the data obtained whether the protein composition (albumins) or the protein properties (TIA, lectins) of the ANF concentrate were responsible. It is important for research on peas to determine whether the implication of pea ANFs in the pea nutritional value is restricted to their biological activity or(and) to their molecular characteristics. Plant breeders will then be able to identify the factors on which they need to concentrate in order to improve the nutritional value of peas. A specific study of pancreatic enzymes, as well as amino acids composition of the ileal digesta, could be an approach to study the role of pea ANFs.

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Chapter IV

AMINO ACID COMPOSITION OF ILEAL DIGESTA COLLECTED FROM PIGLETS FED RAW PEA AND PEA PROTEIN ISOLATE DIETS

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AMINO ACID COMPOSITION OF ILEAL DIGESTA COLLECTED FROM PIGLETS FED RAW PEA AND PEA PROTEIN ISOLATE DIETS

Ileal digesta were collected via a PVTC cannula from piglets (10 - 15kg) fed raw pea (RP) diets and pea protein isolate (PP) diets (Finale or Frijaune variety). From the determined amino acids (AA) content of the feed and the digesta, AA apparent digestibility coefficients and proportions of proteins (endogenous, bacterial, dietary) theoretically present in the digesta were collected. Four essential AA in the two RP diets had a low apparent ileal digestibility: (Frijaune and Finale, respectively) MET (34% and 48%), CYS (47% and 53%), TRP (42% and 48%), THR (63% and 63%). The digestibility coefficients (DC) of these amino acids were higher for the PP diets than for the RP diets: (Frijaune and Finale, respectively) MET (75% and 65%), CYS (61% and 61%), TRP (71% and 68%), THR (79% and 76%). In both cases, they were lower digestible than N.

Proportions of dietary, endogenous and bacterial proteins in the collected digesta were estimated from the AA compositions of the digesta, according to a multiple regression model. The amino acids present in the ileal digesta of piglets fed the RP diets were estimated to originate for 20-30% of them from dietary proteins and for the 70-80% from non-dietary proteins (endogenous and bacteria), for both pea varieties. As for the PP diets, the digesta amino acid composition agrees with a combination of endogenous and bacterial sources.

Amino acids : Ileal digestibility : Peas: Antinutritional factors: Trypsin inhibitors: Piglets

INTRODUCTION

Diets incorporated with high levels of raw pea (more than 30%) exhibit a low N apparent ileal digestibility (Green, 1988; Buraczewska *et al.*, 1989; Huisman *et al.*, 1990; Leterme *et al.*, 1990; Jondreville *et al.*, 1992). However, diets based on pea protein isolates, low in

antinutritional factors (ANFs) and free of pea carbohydrates show a high digestibility (Le Guen *et al*, 1993).

Compared with pea protein isolate digesta, digesta of piglets fed raw peas contain more N, consisting of endogenous N (digestive secretions, cells from the gutwall, bacteria) and (or) of undigested dietary N.

Raw peas contain albumin proteins among which some have antinutritional properties: trypsin inhibitors (TI) and lectins. The results of an earlier study (Le Guen *et al.*, 1993), using pea ANFs supplied in a concentrated form, showed that these compounds can be associated with the low N apparent ileal digestibility of raw pea. The TI could be responsible for a high amount of endogenous N as they can stimulate pancreatic secretions in several animal species (reviewed, Gallaher and Schneeman, 1986).

The objective of the present study was to measure the apparent AA digestibility of raw pea diets and of pea protein isolate diets and to estimate the proportions of dietary and non-dietary proteins in the ileal digesta of piglets fed these diets.

MATERIALS AND METHODS

1. Design

Piglets (10 - 15 kg live weight) fitted with PVTC cannula were used in experiments 1 and 2. These experiments concerned 17 and 18 piglets, respectively. Six diets were formulated with casein+fish meal (control, *C1* and *C2* diets), or with raw peas (spring variety Finale: *RP1* diet, winter variety Frijaune: *RP2* diet), or with pea protein isolates (spring variety Finale: *PP1* diet, winter variety Frijaune: *PP2* diet). The compositions of these diets were given elsewhere (Le Guen *et al.*, 1993).

The experimental procedures were the same as those reported in Le Guen *et al.* (1993), except for the duration of the chyme collection. The results reported in Le Guen *et al.* (1993) corresponded to one period of 5 days. The results reported here concern pooled samples of chyme collected over 2 periods of 5 days (12h) with 2 days rest in between.

2. Chemical analysis

Amino acids, with the exception of methionine, cystine and tryptophan, were determined on an automatic analyser, after acid hydrolysis (HCl 6N for 22 h at 100°C) according to Slump (1969), in the individual ileal chyme samples and in the feeds. For methionine and cystine, prior to this hydrolysis, the samples were submitted to performic acid oxidation. Tryptophan

was determined after hydrolysis with $Ba(OH)_2$ 2.7N at 130°C, according to Slump and Schreuder (1969).

Chromic contents in feed and chyme were determined by hydrolysing the samples in a mixture of perchloric and nitric acids and measuring the Cr^{6+} by flame atomic absorption spectrophotometry.

3. Data analysis

Apparent ileal amino acid digestibility coefficients (DC) were determined from the AA concentrations in the diets and in the digesta samples. The equations are reported in Le Guen *et al.* (1993). When calculating the digestibility of methionine, threonine and tryptophan, the amounts of these synthetic amino acids added to the feed were substracted from the intake by assuming they were completely absorbed in the small intestine (Huisman *et al.*, 1986).
The amino acid patterns of the ileal chyme samples were compared with the amino acid patterns of the chyme samples, of the diets and of various reference proteins. This was done by calculating the chi-square distance as defined by Guilloteau *et al.* (1983).
The reference proteins included:
- pure sources of endogenous proteins present in:
    - new-born piglet meconium from the small intestine (Laplace *et al.*, 1985)
    - germ-free piglet faeces (Laplace *et al.*, 1985),
    - pancreatic juice (Corring and Jung, 1972),
    - pancreatic juice + bile + intestinal secretions (Low and Zebrowska, 1989)
- microbial proteins from isolated fecal bacteria collected from pigs fed a semi-purified diet (Laplace *et al.*, 1985),
- endogenous and microbial proteins present in digesta collected from cannulated pigs of 56 kg fed a N free diet (Darcy-Vrillon and Laplace, 1984),
- dietary proteins:
    - proteins from the feed,
    - pea globulin proteins and their two main fractions: legumin and vicilin (Guéguen, 1990),
    - pea albumin proteins, and pea trypsin inhibitor (Gwiazda *et al.*, 1980).

The chi-square value was calculated from the AA composition of two proteins according to the following formula:

$$(1)\quad \text{distance of } \chi^2 = 17 \sum_{k=1}^{17} \frac{(AAik - AAjk)^2}{\frac{(AAik + AAik)}{2}}$$

where AA_{ik}: % of amino acid k in chyme i
AA_{jk}: % of amino acid k in reference protein j
k: value between 1 and 17

The higher the resulting χ^2 value, the more different are the two proteins being compared. For the calculations, tryptophan was removed from the list of amino acids as it was not always analysed in the reference proteins.

In addition to these two by two comparisons, calculations were done to determine the origin of the proteins present in digesta from RP diets and PP diets. Based on the AA profiles of the digesta and reference proteins, the mathematical model devised by Duvaux *et al.* (1990) was used to estimate the theoretical proportions of reference proteins (ie dietary, endogenous, bacterial) in the digesta. The method combines a multiple regression analysis without constant, and the calculation of the distance of χ^2.
The following equations were solved:

$$(2)\quad \text{digesta} = x_1\,\%\,P_1 + \ldots + x_i\,\%\,P_i + \ldots + x_n\,\%\,P_n \quad \text{where}$$

P_i: reference proteins
x_i: % of P_i present in digesta

The formula was recalculated with different reference proteins until significant regression coefficients x_i were obtained ($p < 0.05$).
As the calculation is based on AA compositions, there are 17 equations to be solved:

$$(3)\quad \begin{vmatrix} AA_1 \\ \vdots \\ AA_i \\ \vdots \\ AA_n \end{vmatrix} \text{digesta} = x_1\,\% \begin{vmatrix} AA_1 \\ \vdots \\ AA_i \\ \vdots \\ AA_n \end{vmatrix} P_1 + x_2\,\% \begin{vmatrix} AA_1 \\ \vdots \\ AA_i \\ \vdots \\ AA_n \end{vmatrix} P_2 + \ldots + + x_n\,\% \begin{vmatrix} AA_1 \\ \vdots \\ AA_i \\ \vdots \\ AA_n \end{vmatrix} P_n$$

minimizing the:

$$(4)\quad \text{distance of } \chi^2 = 17 \sum_{i=1}^{17} \frac{(AAi_{digesta} - AAi_{mixture})^2}{\frac{(AAi_{digesta} + AAi_{mixture})}{2}}$$

where $AAi_{mixture}$: % of amino acid i in mixture of reference proteins

As a first step, 3 categories of proteins were considered for the regression calculations:

- pure sources of endogenous proteins(meconium or axenic piglet faeces or endogenous secretions)
- bacterial proteins
- dietary proteins (feed or pea albumins or pea globulins).

For the RP digesta, the second step consisted in taking into account the PP digesta AA composition, and to find out if bacterial, endogenous or(and) dietary proteins should be added to the PP digesta crude proteins in order to have a significant simulation of the RP digesta crude protein composition.

The significance of differences between treatment means was tested by calculating the variances ratio *F* (Snedecor and Cochran, 1980). All statements of significance are based on a probability of less than 0.05.

RESULTS

Digestibility of amino acids

The variations in each amino acid apparent ileal digestibility coefficient (DC) relatively to nitrogen DC are shown in figure 1.

The average apparent ileal digestibility of the AA was almost identical to that of N (Figure 1). The average DC of AA were 70% and 67% for the RP1 and RP2 diets, respectively, and 83% for both PP diets.

For the RP diets, the AA digestibility coefficients were in the range of 34 to 87% for the essential amino acids (EAA) and 61 to 82% for the non-essential amino acids (NEAA).

Removing the ANFs and the carbohydrates from the raw peas (PP diets) led to a significant increase in apparent ileal digestibility of all amino acids ($p < 0.05$). The values ranged from 61 to 91% for the EAA and from 73 to 93% for NEAA. A few EAA were less digestible in the PP diets (Finale or Frijaune) than in the control diets ($p < 0.05$): LEU (1.4 units), LYS (1.3 units), MET (16.7 and 26.7 units), THR (2.2 units), TRP (12.8 units). Among the EAA, the increases of AA digestibility between the RP diets and the PP diets were not equal for all AA. The highest increases were observed for MET (+41 units for Frijaune, +16 units for Finale) and TRP (+ 29 units for Frijaune, + 20 units for Finale), although for N digestibility the increases were not higher than +14 units for Finale and +15 units for Frijaune.

Some AA had an apparent digestibility much lower than the N digestibility (Figure 1). For the RP diets these AA, in Frijaune and Finale respectively, are: MET (34% and 48%), CYS (47% and 53%), TRP (42% and 48%), THR (63%) among the EAA and PRO (62% and 54%),

GLY (65% and 67%) for the NEAA. The digestibility coefficients of MET, CYS and TRP were lower for Frijaune than for Finale, but higher for PRO. For the PP diets, the AA digestibility coefficients were considerably improved compared to the AA of the RP diets. But the AA still having a lower apparent digestibility than that for N were the same as for RP diets (Frijaune and Finale respectively): MET (75% and 65%), CYS (61%), TRP (71% and 68%), THR (79% and 76%) for EAA and PRO (73%), GLY (82% and 80%)for the NEAA.

Figure 1. *Variation (%) of apparent ileal digestibility coefficients of amino acids of raw pea (RP1, RP2) or pea protein isolate (PP1, PP2) diets fed to piglets of 15 kg, relatively to nitrogen digestibility (N digestibility set at 0)*

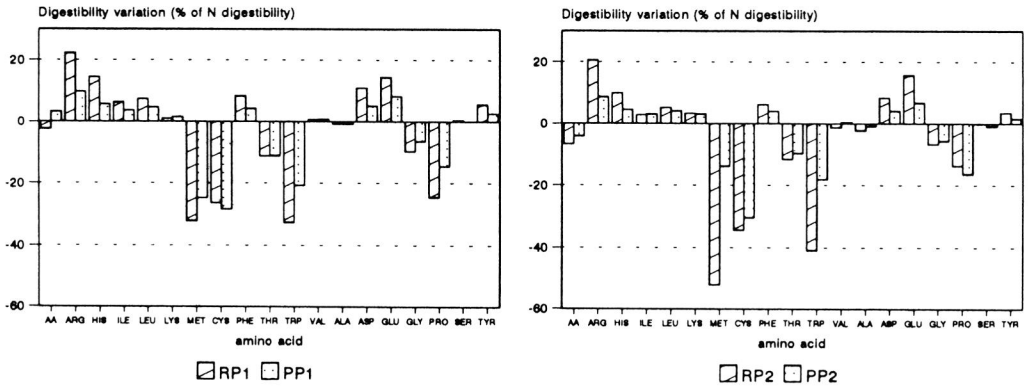

Table 1. *Mean amino acid composition of feed (F) and ileal digesta (D) (each amino acid expressed as % of total amino acids assayed) (experiment 1)*

Diet...	*C1*		*RP1*		*PP1*	
Amino acids	F	D	F	D	F	D
Essential amino acids						
Arginine	4.0	3.3	8.9	4.5	8.6	4.3
Histidine	2.7	2.1	2.9	2.1	2.6	2.1
Isoleucine	5.1	4.3	4.5	4.5	4.8	4.4
Leucine	8.9	6.0	7.6	7.2	8.4	7.2
Lysine	7.0	6.2	6.0	6.8	6.2	6.6
Methionine	3.1	1.8	2.6	1.7	2.9	2.2
Cysteine	0.6	2.2	1.8	2.7	1.0	3.0
Phenylalanine	4.5	3.4	4.8	4.5	5.0	4.4
Threonine	4.2	6.5	4.3	5.8	4.3	6.8
Tryptophan	1.1	1.6	1.1	1.8	1.1	2.0
Valine	6.3	5.6	5.1	5.9	5.2	5.9
Non essential amino acids						
Alanine	3.9	5.2	4.3	5.1	4.1	5.1
Aspartic acid	7.6	12.6	11.7	10.0	11.1	9.2
Glutamic acid	19.7	13.4	17.2	12.9	17.9	10.9
Glycine	3.2	7.0	4.3	6.3	4.0	6.6
Proline	8.4	8.6	4.4	8.3	4.3	9.5
Serine	5.5	7.2	5.3	6.1	5.4	6.4
Tyrosine	4.2	2.8	3.4	3.4	3.3	3.3

Table 2. *Mean amino acid composition of feed (F) and ileal digesta (D) (each amino acid expressed as % of total amino acids assayed (experiment 2)*

Diet...	*C2*		*RP2*		*PP2*	
Amino acids	F	D	F	D	F	D
Essential amino acids						
Arginine	4.2	3.4	9.3	5.3	8.5	4.4
Histidine	4.0	2.1	2.6	2.3	2.5	2.1
Isoleucine	5.0	4.7	4.5	4.9	4.7	4.5
Leucine	8.8	5.9	7.5	7.7	8.1	7.1
Lysine	7.1	5.9	6.0	6.5	6.1	5.9
Methionine	3.2	1.8	3.5	1.9	4.6	1.7
Cysteine	0.6	2.2	1.3	3.0	0.8	3.0
Phenylalanine	4.5	3.2	4.8	4.7	5.0	4.4
Threonine	4.1	6.5	4.4	5.7	5.0	6.6
Tryptophan	1.1	1.5	1.4	1.8	1.4	2.0
Valine	6.3	5.8	5.0	6.0	6.1	6.0
Non essential amino acids						
Alanine	3.9	5.2	4.3	5.4	4.0	5.1
Aspartic acid	7.4	12.5	11.2	10.5	10.9	9.6
Glutamic acid	19.0	14.4	17.1	12.3	17.1	11.4
Glycine	3.0	6.8	4.1	5.7	3.8	6.3
Proline	8.4	9.2	3.9	6.3	3.9	9.7
Serine	5.3	8.0	5.2	6.2	5.2	6.6
Tyrosine	4.2	2.7	3.3	3.6	3.2	3.5

Amino acid composition of ileal chyme

The amino acid compositions of feed and ileal chyme collected in experiments 1 and 2 are given in Tables 1 and 2. The χ^2 distances between chyme compositions in different dietary treatments are given in tables 3 & 4.

The protein present in the ileal chyme was richer in cystine, tryptophan, alanine, glycine and serine than the dietary proteins, irrespective of the dietary protein sources (Tables 3, 4). Similar observations were recorded by Nunes do Prado *et al.* (1989) using veal calves fed three types of diet (skim-milk, pregelatinized pea, soya-bean).

The differences between the two dietary protein sources (casein+fish or pea) are mainly with regard to 5 amino acids, when comparing their concentration in the ileal chyme and in the feed. The aspartic acid concentration in the ileal chyme decreased with pea protein diets (-8 to -15%) but increased with control diets (+70%), compared with the concentration in the feed. The arginine concentration decreased by 50% with pea protein diets and by 15-20% with control diet. The lysine and valine concentrations increased with pea protein diets (+15 to +20% for both amino acids) but decreased with control diets (-8% to -17% for both amino acids). Proline concentration increased by up to 120% with pea protein diets and only by up to 9% with control diets.

Table 3. *Comparison of amino acid profiles (experiments 1 & 2) between chymes of different dietary treatments:* χ^2 *values*

Variety...	within Finale variety (experiment 1)	between varieties	within Frijaune variety (experiment 2)
Dietary treatment:			
C1 *vs.* C2		5	
RP1 *vs.* RP2		17	
PP1 *vs.* PP2		8	
RP *vs.* PP	15		46
RP *vs.* C	38		93
PP *vs.* C	50		50

The results of the two-by-two comparison of the amino acid profiles of the digesta from the various treatments are given as χ^2 distances in Table 3.

The amino acid composition of digesta from the control diets did not change from one experiment to the other, as the χ^2 distance was as low as 5 when comparing C1 digesta to C2 digesta. It indicates the reliability of the experimental procedures.

The results of Table 3 indicate also that the digesta did not have the same AA composition whether the diet was based on animal proteins (casein and fishmeal, C1 and C2) or pea proteins (PP or RP): χ^2 distances from 38 to 93.

The two pea varieties used to produce the PP gave similar digesta AA compositions, as derived from the low χ^2 distance between the digesta AA compositions for PP1 and PP2 diets (8, Table 3). This is confirmed by the values of χ^2 distances between the C and PP digesta which equal to 50 in both varieties (C1 *vs.* PP1, C2 *vs.* PP2).

The pea variety had more effect on digesta AA composition with the RP diets than with the PP diets (χ^2 = 17 for RP1 *vs* RP2 digesta). Similarly, the χ^2 distances differ according to variety when the AA composition of RP digesta are compared with the one of the PP digesta, or the one to the C digesta.

The results of the two-by-two comparison of digesta AA compositions with reference protein AA composition are given in Table 4.

Table 4. *Comparison of chyme amino acid profiles (experiments 1 & 2) with reference proteins:* χ^2 *values*

Variety...		Finale			Frijaune	
Diet...	*C1*	*RP1*	*PP1*	*C2*	*RP2*	*PP2*
reference proteins						
Diets	252	165	277	274	159	320
Pea albumin	154	110	174	180	90	159
Pea globulin	299	240	353	307	200	332
Pea legumin	291	270	377	285	252	343
Pea vicilin	447	376	501	454	336	488
Pea TI	526	543	533	560	535	504
Fecal bacteria	185	135	185	215	76	194
Germ-free piglet faeces	134	124	96	133	154	185
Piglet meconium	158	128	135	188	134	146
Pancreatic juice	141	89	60	158	51	56
Endogenous secretions	145	102	142	171	101	157
0%N-diet-endogenous	95	47	61	117	40	61

As the AA profiles of the digesta differed from the feed AA profiles (Tables 1 and 2), the χ^2 distances between the diets AA compositions and the ileal digesta AA compositions were high (from 159 to 320) (Table 4). The differences between the diet AA profiles and the digesta AA profiles for the RP diet were less than for the other diets. However the similarity was not very high as indicated by the still high χ^2 distances (159 - 165).

For Finale and Frijaune raw pea digesta, the lowest χ^2 distances (< 110) were obtained when compared with bacterial proteins (Frijaune only), pancreatic juice, pea albumins and with endogenous proteins from pigs fed N-free diets (Table 4). The values were influenced by the pea variety when comparing with bacterial proteins (76 for Frijaune and 135 for Finale), with pancreatic juice proteins (51 for Frijaune and 89 for Finale), and with pea albumins (90 for

Frijaune and 110 for Finale). The pea trypsin inhibitor proteins were the ones which differed most from the RP digesta proteins (540).

For PP digesta, the χ^2 distances were the lowest when compared with endogenous secretions (61), pancreatic juice (56 for Frijaune, 60 for Finale) and germ-free piglet feces (85 for Frijaune, 96 for Finale). This means that endogenous were probably the largest source of PP ileal proteins. The proteins present in PP digesta showed no similarity with any of the pea proteins. This is consistent with the high χ^2 distances between the feed and the digesta AA profiles (277 and 320).

For the control diets, the lowest χ^2 distances found were 95 and 117 when compared with the proteins present in digesta from pigs fed a N free diet (Table 4).

Origin of the undigested proteins

The results of the best multiple regressions according to equation 3 are given in table 5 for the RP digesta and in table 6 for the PP digesta. For the RP digesta, the results are divided in 3 parts: 3 categories of separate protein sources (pure endogenous, bacterial, dietary), 3 categories of overlapping protein sources (pure endogenous+bacterial, bacterial, dietary), 4 categories of protein sources (PP digesta proteins + separate protein sources).

The χ^2 distances calculated from the results of the multiple regressions indicate that RP digesta amino acids originated from several proteins and not one particular protein. The χ^2 values ranged from 4 to 32 for the best regressions reported in table 5 whereas the lowest χ^2 values found by the two by two comparison for the RP digesta were 40 and 47 (table 4).

The proteins from which the RP digesta AA would theoretically originate from, would depend on the pea variety, in agreement with the results of table 3.

For Finale variety, the digesta amino acids would come from 25-30% pea albumins, 30-45% bacterial proteins and 25-35% pure endogenous proteins, with a close fit (coefficient of determination R^2 of 0.80-0.84) and low χ^2 distances (15-18) (see *1.1. a,b,c* in Table 5). When it was assumed that the dietary proteins were the proteins from the feed, the statistical values of the estimation were not as reliable as when the albumins were considered as the dietary proteins. The proportions of dietary proteins, and therefore also of the non-dietary proteins, were similar whatever the source of endogenous protein considered. The different sources of the endogenous protein (meconium, endogenous secretions or germ-free piglet feces) influenced the estimated proportions of each of the non-dietary proteins (bacterial or pure endogenous, see *1.1(a,b,c)* table 5). If the endogenous proteins used in the model were not the "pure" endogenous but a mixture of endogenous and bacterial proteins of digesta from pigs fed N-free diet, the RP1 digesta amino acids would then have originated from 28% albumins, 43% of these endogenous proteins but less bacteria (25%) (see *1.2* in Table 5). If PP1 ileal digesta amino acids were considered in the calculations along with the 3 categories

Table 5. *Proportions of proteins (%) from various sources that would match, according to equation 3, the amino acid composition of ileal digesta from pigs fed Finale (RP1) or Frijaune (RP2) raw peas*

Protein sources	Calculated proportions	SE	Significance (P)	
		FINALE raw pea digesta (RP1)		
1.1(a) - meconium	25.5%	0.075	0.004	
- bacteria	38.5%	0.098	0.001	
- albumins	31.2%	0.111	0.013	$R^2=0.81$
(b) - endo. secretions	34.3%	0.107	0.006	
- bacteria	31.7%	0.108	0.010	
- albumins	29.4%	0.117	0.024	$R^2=0.80$
(c) - germ-free lamb faeces	23.2%	0.059	0.001	
- bacteria	44.9%	0.089	0.000	
- albumins	27.2%	0.106	0.021	$R^2=0.84$
1.2 - 0%N-diet-endogenous	42.5%	0.104	0.001	
- bacteria	24.8%	0.101	0.026	
- albumin	27.7%	0.103	0.016	$R^2=0.85$
1.3(a) - PP1 digesta	19.2%	0.086	0.041	
- endo. secretions	22.6%	0.107	0.052	
- bacteria	28.8%	0.095	0.008	
- albumin	25.1%	0.104	0.030	$R^2=0.86$
(b) - PP1 digesta	18.0%	0.090	0.063	
- meconium	16.6%	0.081	0.057	
- bacteria	33.8%	0.092	0.002	
- albumin	27.0%	0.102	0.018	$R^2=0.86$
		FRIJAUNE raw pea digesta (RP2)		
2.1(a) - meconium	29.0%	0.009	0.006	
- bacteria	47.2%	0.137	0.004	
- dietary protein	21.9%	0.114	0.075	$R^2=0.61$
(b) - endo. secretions	38.6%	0.141	0.015	
- bacteria	46.8%	0.152	0.008	
- dietary protein	12.7%	0.130	0.345	$R^2=0.56$
(c) - germ-free lamb faeces	34.0%	0.051	0.000	
- bacteria	50.3%	0.080	0.000	
- dietary protein	14.8%	0.074	0.063	$R^2=0.84$
2.2 - 0%N-diet-endogenous	56.9%	0.104	0.000	
- bacteria	23.0%	0.123	0.080	
- dietary protein	18.7%	0.085	0.044	$R^2=0.79$
2.3(a) - PP2 digesta	39.6%	0.058	0.000	
- pancreatic	18.4%	0.074	0.025	
- bacteria	26.1%	0.077	0.004	
- dietary proteins	15.8%	0.045	0.003	$R^2=0.95$
(b) - PP2 digesta	47.0%	0.065	0.000	
- meconium	6.5%	0.051	0.220	
- bacteria	31.2%	0.066	0.000	
- dietary protein	15.4%	0.052	0.010	$R^2=0.93$

R^2: coefficient of determination of the regression model. SE: standard error of the estimate.

of proteins (pure endogenous, bacterial, dietary), these digesta amino acids would be present at a level of 19% (p=0.04). In addition, there would still be 25% albumins, 29% bacterial proteins and 23% endogenous secretions (R^2 = 0.86 and χ^2 = 12) (see *1.3(a,b)* in Table 5). For Frijaune variety, the digesta amino acids would come from 22% feed proteins, 47% bacterial proteins and 29% meconium (R^2 = 0.61 and χ^2 = 32) (see *2.1 a* in Table 5). In the RP2 digesta, the feed proteins would be in most cases significantly present but not the pea albumins as for the RP1 digesta. If "pure" endogenous proteins from endogenous secretions or from germ-free piglet faeces were considered instead of meconium proteins, the RP2 digesta amino acids would come less from dietary proteins (13 to 15%) and more from non-dietary proteins (about 85%) (20 to 33%) (see *2.1 b,c* in Table 5). If the endogenous proteins from pigs fed N-free diet were considered in the regression, the digesta AA origins would be 19% feed proteins, 23% bacterial proteins and 57% of these endogenous proteins (R^2 = 0.79 and χ^2 = 17) (see *2.2* in Table 5). If the PP2 digesta AA profile was considered in the model, these proteins would constitute 40 to 47% of the RP2 digesta AA. In addition, there would be 25-30% bacterial proteins, 18% pancreatic juice or 6 meconium and 15% feed proteins (R^2 = 0.93 - 0.95 and χ^2 = 4 - 6) (see *2.3 a,b* in Table 5).

The PP digesta amino acids (Finale, Frijaune) would not originate from numerous proteins unlike the RP digesta amino acids. Combining 3 or 4 categories of proteins as for the RP digesta (Table 6) did not resulte in significant regression model. Some models reported in table 6 indicate that the PP digesta AA would originate mostly from endogenous proteins.

Table 6. *Proportions of proteins (%) from various sources that would match, according to equation 3, the amino acid profiles of ileal digesta from pigs fed Finale (PP1) or Frijaune (PP2) pea protein isolate*

Protein sources	Calculated proportions	SE	Significance (P)	
PP1 ileal digesta				
- meconium	48.6%	0.146	0.005	
- bacteria	46.2%	0.146	0.006	R^2=-0.03
- meconium	-12.7%	0.236	0.600	
- pancreatic juice	10.0%	0.237	0.678	
- 0%N-diet-endogenous	98.8%	0.385	0.021	R^2=0.37
PP2 ileal digesta				
- meconium	45.8%	0.151	0.008	
- bacteria	47.5%	0.151	0.007	R^2=-0.03
- meconium	-20.7%	0.227	0.605	
- pancreatic juice	12.3%	0.232	0.377	
- 0%N-diet-endogenous	103.7%	0.375	0.014	R^2=0.46

R^2: coefficient of determination. SE: standard error of the estimate.

DISCUSSION

Digestibility of amino acids

Five amino acids from the raw pea diets had a low apparent ileal digestibility: MET (34% and 48%), CYS (47% and 53%), TRP (42% and 48%), THR (63%) and PRO (62% and 54%). Apparent DC of MET, CYS and THR, lower than DC of N and total AA were also found by Combe *et al.* (1988) in rats fed peas. The low apparent digestibility of THR and PRO could be due to high concentration of THR and PRO in endogenous secreta reaching the end of the ileum (Leterme *et al.*, 1990). Jondreville *et al.* (1992) reported apparent ileal digestibility coefficients of 70% for MET, 53% for CYS, 47% for TRP, 60% for THR and 72% for N, measured for the Frijaune pea. For the Solara spring pea, they found higher values than in the present experiment: 81% for MET, 71% for CYS, 70% for TRP, 76% for THR and 80% for N. On the other hand, Leterme *et al.* (1990) measured lower values than Jondreville *et al.* (1992) did for Solara pea: 78% for MET, 64% for CYS, 58% for TRP, 66% for THR, 70% for PRO and 74 % for N. The differences in amino acid digestibility coefficients reported in various studies, especially for MET, could be due to several factors. The age difference of the animals could be one factor. In the present experiments, the pigs were about 15 kg live weight whereas in the study by Jondreville *et al.* (1992), the pigs weighed 40 to 60 kg, and that by Leterme *et al.* (1990) the animals weighed 50 kg. Young animals would be more sensitive than older ones to raw pea diets (pigs; Grosjean and Gatel, 1986) or to raw soybean diets (chickens; Saxena *et al.*, 1963).

The method of chyme collection (ileal-rectal anastomosis or cannulation technique) could also partly explain the differences. Leterme *et al.* (1990) and Köhler *et al.* (1992) showed significant differences between these different techniques with regard to the digestibility of some essential amino acids.

The level of crude protein in the diet is another important element to take into account when comparing apparent digestibility coefficients. The relative importance of endogenous N in the ileal chyme N is minimized when the crude protein level in the diet is higher than 16% (Dammers, 1964). Finally the large variation in composition between varieties of peas of the same genotype could lead to different nutritional values as suggested by Buraczewska *et al.* (1989) and Gdala *et al.* (1991).

The difference in apparent digestibility of amino acids between RP and PP diets could be due to ANFs, as shown on N digestibility in Le Guen *et al.* (1993), but also due to carbohydrates. Conversely to PP diets, the RP diets contain pea pectins and pea cell walls. Pectins could have stimulated endogenous secretions and(or) reduced reabsorption of endogenous AA in the ileum (de Lange *et al.*, 1989), and reduced the apparent ileal digestibility of amino acids as observed by Dierick *et al.* (1983). Similarly, the presence of non-purified fibres in the RP

diets may have decreased re-absorption of endogenous AA in the small intestine and(or) increased mucus production (de Lange *et al.* 1989). Purified cellulose, as in PP diets, would have little effect on N and AA ileal apparent digestibility in pig diets (Dierick *et al.*, 1989; Furuya and Kaji, 1991; Leterme *et al.*, 1992).

Origin of the undigested proteins

Ileal digesta contain free, peptide-bound and protein-bound amino acids (Low and Zebrowska, 1989; Souffrant, 1991), the latter fraction being the largest in the total endogenous N in ileal digesta (Moughan and Schuttert, 1991). The origins of the ileal AA can be dietary proteins not fully hydrolysed or residues of proteins secreted by the digestive tract and organs (pancreatic and intestinal secretions, bile) or proteins of the intestinal microbial population. The proportions of these different proteins are dependent on the diets.

Multiple regression analysis were performed based on AA composition of reference proteins to investigate the origins of the amino acids found in digesta of pigs fed raw peas or pea protein isolate.

The amino acids in the RP digesta would be for 20 - 30% from dietary proteins, according to the various calculations performed. The N apparent ileal DC of the RP diets were found to be 67 - 70%. Considering the calculated proportion of dietary amino acids in the digesta, the N true ileal digestibility for the RP diets would be approximately 90 - 95%, in agreement with the results of Huisman *et al.* (1992) obtained in an isotopic dilution (N_{15}) experiment using young piglets and the same RP diets.

The dietary proteins escaping digestion in the small intestine would be mainly pea albumin proteins for the Finale RP diets. It is consistent with the assumption made in Le Guen *et al.* (1993), regarding the low ileal digestibility of pea albumins. Gatehouse et al. (1985) have shown the resistance of albumins in the pea seed to hydrolysis during germination. The low digestibility of cystine, methionine and tryptophan amino acids found for the RP diets could be partially due to the presence of resistant albumins in the digesta. The two main iso-albumins in peas represent only 5 to 10% of the total pea proteins but contain 25% of the total sulfur amino acids (Guéguen and Baniel, 1990). Pea albumins contain also 2.2 times more tryptophan than the pea globulins which account for 60% of the pea proteins. The low true ileal digestibility of TRP and CYS (Buraczewska *et al.*, 1989; Leterme *et al.*, 1990; Gdala *et al.*, 1992) may be associated with the poor digestibility of albumins.

For Frijaune RP diets, based on the calculation models, the dietary proteins escaping digestion would not be specially albumin proteins, unlike Finale. The reasons are not clear. One explanation could have been the higher trypsin inhibitor activity of the RP2 diet leading to a poor digestion of some dietary proteins. The proportions of undigested dietary proteins in the digesta of the Frijaune and the Finale RP diets have been found to be similar according to the

calculations. The Frijaune albumins probably had also a low digestibility as the cystine, methionine and tryptophan DC were low, as for Finale pea, and the χ^2 distance between pea albumins and Frijaune RP digesta was low (Table 4).

The RP digesta amino acids would originate for 70 - 80% of them from non-dietary proteins. According to the calculations, these 70 - 80% non-dietary proteins would consist in 30 - 40% bacterial proteins, or even 45 to 50% in some cases (Table 5), and 25 to 35% pure endogenous proteins. The model is mathematically coherent because entering endogenous proteins from digesta of pigs fed N-free diet (endogenous = pure endogenous + bacteria) in the model results in a lower calculated proportion of bacterial proteins (*1.2* and *2.2* in Table 5). To consider these results it has to be assumed that endogenous N from digesta of pigs fed N-free diet (Darcy-Vrillon and Laplace, 1984) has the same AA composition as the endogenous N from digesta of pigs fed dietary proteins, although this can be argued (Butts *et al.*, 1993).

However the proportions of bacterial N in the small intestinal chyme seem slighlty high in some cases. Proportions of 25 - 30% were reported by Poppe *et al.* (1983), Drochner (1984), and Wünsche *et al.* (1991). Dierick *et al.* (1983) calculated bacterial N proportions of 30% to 45% of ileal digesta N collected from pigs weighing 30kg, fed different types of diets. In the present study, a high proportion of bacterial N could be related to the fact that pea starch would not be entirely digested by pigs enzymes at the ileal level (Bengala Freire *et al.*, 1991; Leterme *et al.*, 1990), especially starch from winter pea. The RP diets contain also α-galactosides that are not hydrolysed in the small intestine as the piglets are not equipped with adequate enzymes. These carbohydrates may be a source of substrates for the microbial population in the small intestine (Saini, 1989). Pea pectins may also stimulate bacterial activities (Costa *et al.*, 1989; Lupton and Marchand, 1989)

In conclusion, the results of the present study indicate that three essential amino acids of the raw peas had particularly low apparent ileal digestibility coefficients (<50%): MET, CYS and TRP. According to multiple regression analysis, 20 - 30% of the amino acids present in the ileal digesta of piglets fed raw peas would come from undigested pea proteins, possibly albumin fraction. This was in agreement with results obtained with ^{15}N dilution technique. Conversely to raw pea digesta, pea protein isolate digesta would not contain dietary amino acids. When the piglets were fed raw peas, it was calculated that a high proportion of non-dietary amino acids was excreted in the ileal chyme. Measurements of pancreatic protease activity in chyme, pancreas and pancreatic secretions could be used to investigate this non-dietary origin, especially in relation to protease inhibitor intake.

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Chapter V

EFFECT OF PEA ANTINUTRITIONAL FACTORS ON PANCREATIC PROTEASE ACTIVITY IN THE INTESTINAL CONTENT OF PIGLET DIET

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EFFECT OF PEA ANTINUTRITIONAL FACTORS ON PANCREATIC PROTEASE ACTIVITY IN THE INTESTINAL CONTENT OF PIGLET DIET

The effects of dietary pea antinutritional factors (ANFs) on proteolytic enzyme activities in the small intestine of piglets were investigated. Piglets (10 - 15 kg bodyweight) fitted with a PVTC cannula, were fed diets based on raw peas (RP) or pea protein isolate (PP) with or without addition of pea ANF concentrates. Activities of trypsin (T) and chymotrypsin (CT) in digesta removed from the upper jejunum and from the end of the ileum were measured. The addition of ANF concentrate to the PP diet led to decreased activities in the jejunal chyme (-50% for T, -45% for CT). The effect was of the same magnitude at 1 day and 9 days of feeding. In the ileal chyme, the activities were higher for the ANF enriched PP diets (+160% for T, +144% for CT). When the diets were based on raw peas, the activities of T in the ileal chyme, were higher than for the PP diet (+325%) and ANF enriched PP diets (+65%). For CT, the effects were in most cases not significant. The addition of ANF concentrate to the RP diet did not affect the protease activities measured in the ileal chyme. Protease activities at the end of the small intestine were dependent on the diet composition (RP *vs.* PP) and did not reflect the enzyme levels in the upper intestine.

Pancreatic enzymes: Trypsin: Chymotrypsin: Activity: Intestine: Jejunum: Ileum: Chyme: Peas: Antinutritional factors: Trypsin inhibitors: Piglets

INTRODUCTION

Several investigators have reported that raw peas (*Pisum sativum*) fed to piglets have low nitrogen (N) or amino acid apparent ileal digestibility coefficients (Green, 1988; Buraczewska *et al.*, 1989; Huisman *et al.*, 1990; Leterme *et al.*, 1990; Jondreville *et al.*, 1992). However pea protein isolates, containing low levels of antinutritional factors (ANFs) and no carbohydrates, had a high N apparent ileal digestibility (Le Guen *et al.*, 1993*a*). When pea ANF concentrates were added to the pea protein isolate, the protein digestibility was decreased (Le Guen *et al.*, 1993*a*). From these findings it was concluded that pea

proteinaceous ANFs, containing protease inhibitors (PI) and lectins, can influence N apparent ileal digestibility. The effect of pea PI on digestibility could be due to stimulation of enzyme secretion from the pancreas similar to soybean PI (reviewed by Gallaher and Schneeman, 1986). Previous reports indicate changes in pancreatic proteases activity in intestinal chyme upon feeding soybean PI (Kai *et al.*, 1984, rats; Berg Lea *et al.*, 1989, fish; Haisdai *et al.*, 1989, guinea pigs; Gorrill and Nicholson, 1971, calves; Yen *et al.*, 1977, pigs).
The aim of the experiments reported here was to determine the effects of dietary pea ANFs on pancreatic protease activity in relation to different diets, as measured in the upper jejunum and in the proximal ileum.

MATERIALS AND METHODS

1. Design

The activities of trypsin and chymotrypsin were measured in the digesta collected at the end of the ileum from the piglets used in experiments 1 and 2 (Le Guen *et al.*, 1993*a*), and in digesta collected from the upper jejunum in piglets fitted with jejunal cannula (experiment 3).

The diet compositions have been previously extensively described (Le Guen *et al.*, 1993*a*). The main components of the diets are reported in table 1.
The trypsin inhibitor activities and lectin contents of the feed are given in table 1.

Table 1. *Feed : brief composition (g/100g), trypsin inhibitor activity and lectin contents*

Experiment	1		2, 3		2		
Diets...	*PP3*	*PP3*$^{+}$	*PP4*	*PP4*$^{++}$	*RPP4*	*RPP4*$^{+}$	*RPP4*$^{++}$
PP (Finale)	18.4	16.4	-	-	-	-	-
Commercial PP	-	-	15.0	15.0	9.3	9.3	9.3
PP (50%Fi+50%Fr)	-	-	4.0	4.0	2.4	2.4	2.4
Raw pea Solara	-	-	-	-	30.0	30.0	30.0
Pea ANF conc.$^{\text{batch a}}$	-	2.9	-	-	-	-	-
Pea ANF conc.$^{\text{batch b}}$	-	-	-	0.6	-	0.4	0.6
Maize starch	52.2	51.3	51.4	50.8	29.2	28.6	28.8
Dextrose	15.0	15.0	15.0	15.0	15.0	15.0	15.0
TIA[a]	0.1	1.2	0.1	0.8	0.4	0.9	1.1
Lectins[b]	0.5	2.7	1.5	1.9	1.9	2.5	3.4

RP: raw pea; *PP*: pea protein isolate. *Fi, Fr*: spring pea Finale and winter pea Frijaune. Solara: spring pea
conc.: concentrate of pea ANF, batch a: 50% Fr + 50% Fi, batch b: 100% Fr
[a] TIA, trypsin inhibitor activity; in mg inhibited trypsin/g feed.
[b] Lectins: in mg/g feed.

2. Experimental procedures

The animals used in the experiments 1 and 2 were fitted with a PVTC cannula that allowed for digestibility measurements (Le Guen *et al.*, 1993a) and for enzyme determinations in the ileal chyme as reported here. The experimental procedures concerning the animals and the feeding were previously described (Le Guen *et al.*, 1993*a*).
The experiment 3 was designed specifically for jejunal chyme collection.

Pre-experimental procedures for experiment 3
Piglets (15 total, 11 kg live weight) of the same origin as those used in experiments 1 & 2, were housed individually in cages. After one week of cage adaptation, they were surgically fitted with a simple T cannula in the jejunum, 1 meter after the ligament of Treitz. These surgical procedures were based on those for cannulation at the terminal end of the ileum developed by van Leeuwen *et al.* (1990). They differed only in the way the cannula was introduced. After localisation of an insertion point in the upper jejunum, a small incision was made to allow the insertion of the T cannula. Surgical suture was used to tighten the jejunal mucosa around the cannula. An opening to exteriorize the cannula was made below the ribs, in the right side of the abdomen. The cannula were closed and secured. The piglets were allowed to recover for one week before they received the experimental feed. At the end of the recovery week, chyme was collected over a period of 6 h to allow an adaptation to the collection procedure.
The feed was given at 08.00 and 16.00 hours, as pellets mixed with water (1:2, weight) at a restricted level of 2.6 times the maintenance energy requirements (ARC, 1981). The experimental feed was introduced on the first day of the chyme collection period (cf. following paragraph).

Chyme collection procedures (all experiments)
In experiment 1, 10% of ileal chyme collected hourly from 12.00 to 14.00 hours (meal given at 08.00 hours) were sampled and immediately stored at -20°C. This procedure was repeated during 5 consecutive days (day 8 to day 12 (D8-D12); day 1 being the first day the experimental diets were given). After freeze-drying, the chyme samples per animal were pooled together.
In experiment 2, the total amount of ileal chyme produced between 10.00 hours and 15.00 hours (meal given at 08.00 hours) was collected per hour on day 9 (day 9 D9; day 1 being the first day the experimental diets were given). The chyme was immediately frozen and then freeze-dried.

In experiment 3, the jejunal chyme was collected on day 1 and day 2 (D1-D2); day 1 being the first day the experimental diets were given, and on day 8 and day 9 (D8 - D9). The chyme was collected in plastic bags during 7 sessions of 15 minutes each, every half-hour, from 08.00 h until 13.00 h. At the end of each session, the cannula were closed, the chyme was weighed and frozen in a pot specific for the day and for each animal. Prior to chemical analysis, the chyme collected per animal was thawed and kept cold, pooled (D1 and D2 together, D8 and D9 together), homogenized, sub-sampled and freeze-dried.

3. Chemical analysis

A "freeze-drying" dry matter content was determined by freeze-drying the chyme to constant weight (experiments 1 and 2). In experiment 3, an "oven" dry matter was determined by drying in the oven a wet subsample of the chyme.

Trypsin inhibitor activity was determined using the Kakade method as modified by van Oort *et al.* (1989). Lectin content was measured according to an ELISA technique (Hamer *et al.*, 1989).

Trypsin (T) (EC 3.4.21.4) and chymotrypsin (CT) (EC 3.4.21.1) activities were determined in the freeze-dried chyme, using p-toluenesulfonyl-L-arginine methyl ester (TAME) and benzoyl-L-tyrosine ethyl ester (BTEE), respectively, as substrates, on a spectrophotometer (Perkin Elmer 550), without activating the zymogens (Bergmeyer, 1974). Activities are expressed as units (U), one unit being defined as the amount of enzyme which hydrolyses one μmol substrate per minute under the assay conditions (T=25°C, pH=8.1 for T and 7.8 for CT).

4. Calculations and statistical procedures

A one-way analysis of variance (software package SPSS/PC, Norusis, 1988) was carried out per experiment, and per sampling day (day D1-2, day D8-9) for experiment 3, using the following model:

$$Y_{ij} = \mu + D_i + e_{ij}$$

where Y_i = dependent variable, μ = mean, D_i = diet effect and e_i = residual error between animals. For experiments 1, 3(D1-2) & 3(D8-9): i = 1, 2 and for experiment 2: i = 1 .. 5.

The results are given as means with their standard errors.

RESULTS

Trypsin (T) and chymotrypsin (CT) activities in the intestinal content are expressed as per g of wet or dry digesta (specific activity) and in the total amount of digesta collected (total activity).

The data related to chyme collected in the upper jejunum (experiment 3) are shown in table 2. The amounts of chyme, as wet or dry, were not influenced by the diets or the day of collection. Addition of pea ANF concentrate to PP4 diet decreased T and CT activities (specific and total) in the jejunal chyme ($p < 0.05$). The T to CT ratios were not changed by the addition of ANF concentrate. Enzyme activities were not affected by the day of collection.

Table 2. *Trypsin and chymotrypsin activity (specific and total) in the jejunal chyme collected during 1h45, from 0 to 5 h p.p., from piglets fed pea diets: mean and (standard error)*

	Days 1 and 2		Days 8 and 9	
Diets...	*PP4*	*PP4++*	*PP4*	*PP4++*
n observations	6	6	6	6
Feed (g/meal)	274	273	274	273
Total chyme collected	*during 1h45, from 0 to 5 h p.p.*			
wet (g)	915[a]	901[a]	1012[a]	934[a]
	(39)	(72)	(155)	(212)
% DM	17.0[a]	17.5[a]	16.3[a]	17.2[a]
	(.5)	(.3)	(.6)	(.5)
dry (g)	155[a]	159[a]	161[a]	159[a]
	(5)	(14)	(9)	(13)
Specific activity (U/g wet chyme)				
trypsin	21.5[a]	10.8[b]	24.6[a]	16.1[b]
	(2.4)	(.7)	(2.1)	(.8)
chymotrypsin	1.6[a]	0.9[b]	1.4[a]	1.0[b]
	(.2)	(.2)	(.1)	(.1)
Specific activity (U/g dry chyme)				
trypsin	128[a]	62[b]	151[a]	94[b]
	(15)	(4)	(10)	(6)
chymotrypsin	9.3[a]	5.3[b]	8.8[a]	6.0[b]
	(1.1)	(1.1)	(.3)	(.7)
Ratio Trypsin/Chymotrypsin	14.1[a]	12.8[a]	17.4[a]	16.3[a]
	(1.3)	(1.8)	(.7)	(1.9)
Total activity (U in wet chyme)				
trypsin	19730[a]	9789[b]	24934[a]	15239[b]
	(2479)	(1119)	(2839)	(1844)
chymotrypsin	1435[a]	810[b]	1424[a]	945[b]
	(176)	(103)	(123)	(100)

p.p.: post-prandial hours.

Unit: quantity of enzyme needed to hydrolyse one μmoles of substrate T.A.M.E. (trypsin) or B.T.E.E. (chymotrypsin) per minute in the assay conditions.

Values within day1+2 or day8+9 and line not followed by a similar letter differ significantly ($p < 0.05$).

Table 3. *Trypsin and chymotrypsin activity (specific and total) in the ileal chyme collected from 2 to 7 h p.p., from piglets fed pea diets: mean and (standard error)*

Experiment...	1		2				
Diets...	*PP3*	*PP3+*	*PP4*	*PP4++*	*RPP4*	*RPP4+*	*RPP4++*
n observations	5	5	4	6	5	6	5
Feed (g/meal)	279	279	238	237	237	238	240
Total chyme collected				*from 2 to 7 h p.p.*			
g wet	-	-	83^{a}	101^{a}	197^{b}	190^{b}	195^{b}
	-	-	(6)	(12)	(17)	(22)	(29)
g dry	-	-	15^{a}	15^{a}	30^{b}	27^{b}	25^{b}
	-	-	(.5)	(1.3)	(1.8)	(1.6)	(1.3)
Specific activity (U/g wet chyme)							
trypsin	24.7^{a}	51.6^{b}	25.1^{a}	51.7^{b}	45.0^{ab}	55.8^{b}	46.2^{ab}
	(2.9)	(4.1)	(7.6)	(5.8)	(6.5)	(9.2)	(4.7)
chymotrypsin	8.0^{a}	9.1^{a}	9.9^{ab}	18.0^{c}	7.3^{ab}	10.6^{b}	5.9^{a}
	(1.0)	(.8)	(.6)	(1.4)	(1.0)	(1.8)	(.8)
Specific activity (U/g dry chyme)							
trypsin	-	-	140^{a}	360^{b}	288^{b}	360^{b}	349^{b}
	-	-	(37)	(47)	(42)	(34)	(17)
chymotrypsin	-	-	57^{ab}	122^{c}	47^{a}	69^{b}	47^{ab}
	-	-	(6)	(7)	(6)	(7)	(12)
Ratio Trypsin/Chymotrypsin							
	3.2^{a}	5.8^{b}	2.6^{a}	2.9^{a}	6.2^{b}	5.3^{b}	8.4^{c}
	(.9)	(1.0)	(1.6)	(.7)	(1.3)	(.3)	(2.6)
Total activity (U in wet chyme)							
trypsin	-	-	2001^{a}	5165^{b}	8492^{c}	9865^{c}	8597^{c}
	-	-	(999)	(1904)	(2568)	(2683)	(1674)
chymotrypsin	-	-	824^{a}	1764^{bc}	1373^{abc}	1874^{c}	1212^{ab}
	-	-	(153)	(499)	(275)	(510)	(820)

p.p.: post-prandial hours. Values within experiment and line not followed by a similar letter differ significantly ($p < 0.05$). Unit: quantity of enzyme needed to hydrolyse one μmoles of substrate T.A.M.E. (trypsin) or B.T.E.E. (chymotrypsin) per minute in the assay conditions.

Table 3 shows the results of enzymatic activities in the chyme collected at ileo-caecal level (experiments 1 and 2). The amounts of ileal chyme were higher when the diets were based on RP instead of PP. The amounts of chyme were not affected by the addition of ANF concentrate.

The T activities in ileal chyme were the lowest for the PP diets. This was not the case for CT activities. The addition of 2.9% ANF concentrate (batch a) to the PP diet (experiment 1) increased specific activity of T by 109%. CT activity remained unchanged. In experiment 2, the addition of 0.6% of ANF concentrate (batch b) to the PP diet increased T and CT specific activities by 106% and 82% respectively, and T and CT total activities by 158% and 114% respectively.

The specific T activity in the chyme of the piglets fed the RP diet was similar to the activity in the chyme of those fed the PP+ANF diet, but the CT activity was lower. The addition of

0.4% or 0.6% of ANF concentrate to the RP diet did not significantly affect specific and total T activity.

The ratios of T to CT show two classes of data (table 3): above 5.0 for the raw pea based diets and the PP diet enriched with 3% ANF concentrate, and below 5.0 for the pea protein isolate based diets.

DISCUSSION

The results of the present study demonstrate that addition of pea ANF concentrate to semi-synthetic diets fed to piglets can alter the pancreatic protease activities in chyme of the gastro-intestinal tract. This effect would depend on whether the diets are based on raw pea or on pea protein isolate. In literature, no data are available on the specific effects of an addition of pea PI on protease activities in pigs. The reported data concern mainly soybean trypsin inhibitors and show large variations in their effects on enzymatic activity. For instance, in pigs, the addition of soybean trypsin inhibitors (SBTI) to heated soybean meal (HSBM) diets decreased protease activities in the ileal chyme (Yen *et al.*, 1977) but had no effects when added to casein diet (Schulze *et al.*, 1992). Struthers *et al.* (1983) measured lower trypsin activity in feces of piglets when they were fed a raw soybean meal diet rather than HSBM or casein diets.

In the present study, the results of protease activities in the chyme corresponding to diets based on pea proteins differed according to the site of chyme sampling.

Proteases in jejunal chyme - PP diet

T and CT activities measured in the jejunal chyme decreased when 0.6% ANF concentrate was added to the PP diet. Hasdai *et al.* (1989) also found lower protease activities in the jejunal chyme of guinea-pigs fed a raw soybean flour diet compared with those fed a heated soybean flour diet. The effect of ANFs on protease activities was obvious in 1 or 2 days in the present study, consistant with observations made by Yen *et al.* (1977). The effect was then steady over the rest of the feeding period. The procedures of jejunal chyme collection itself did not probably affect the feedback regulation of pancreatic secretion to a large extent as the regulation mainly occurs in the duodenum (Corring, 1974). It needs to be stressed here that the sampling through the T cannula has to be homogeneous. We observed that the dry matter content and the amounts of chyme that were collected were similar between days.

The lowered protease activity in the jejunal chyme of piglets fed the PP diet enriched with pea ANF concentrate, as observed in the present study, may be due to the inhibition of the

proteases by the pea PI. The decrease of T and CT activities due to PI may have been partly amplified by the level of intraluminal enterokinase. In rats, Kai *et al.* (1984) observed a positive correlation between the intraluminal trypsin activity and the intraluminal enterokinase activity, the latter depending on the former. If the intraluminal trypsin activity is decreased, the enterokinase levels would then decrease and therefore less trypsinogen would be activated. According to the feedback mechanism, often referred to in studies using rats fed SBTI (Gallaher and Schneeman, 1986), one might have expected an increased secretion of enzymes from the pancreas to compensate for the inhibited enzymes. Schneeman *et al.* (1977), Roy and Schneeman (1981) and Temler *et al.* (1984) observed, with rats and mice, that protease activity in the pancreas or its secretions were increased when SBTI were added to a casein-based diet. Green and Nasset (1983) also measured increased secretions of pancreatic enzymes with "casein+lima bean TI" in rats. Laporte and Trémolières (1973) observed an elevation of trypsin secretion from the pancreas of rats when PI were infused in the duodenum. Gertler and Nitsan (1970) observed the same trend with a HSBM + SBTI diet, in chicks. However, in our studies, the enzyme activities measured in the jejunal chyme decreased when pea ANF concentrates were added. Birk and Smirnoff (1992) observed no modification of synthesis and secretions of pancreatic enzymes in chicks fed chick pea protease inhibitors. Similarly, addition of SBTI to HSBM diet (Yen *et al.*, 1977) or to a RSBM diet (Corring *et al.*, 1986) did not increase enzyme contents in the pancreas of piglets. Struthers *et al.* (1983) observed decreased pancreatic T and CT activity in the pancreas of piglets fed RSBM compared to casein or HSBM.

From the present study, it cannot be derived whether more proteases were secreted into the intestinal lumen. On one hand, the pancreatic secretion of enzymes may have increased. However to have less free proteases in the jejunal chyme, all the extra proteases secreted by the pancreas would then have been bound to the dietary PI. The pancreas would thus be unable to restore the normal concentration of proteases in the intestine. On the other hand, the pancreas may have not been stimulated to produce more proteases. In that case, the remaining free T and CT activities in the jejunal chyme after partial inhibition by PI may have been sufficient for the hydrolysis of the PP diet proteins, highly digestible in the small intestine as shown before (Le Guen *et al.*, 1993*a*).

Proteases in ileal chyme - PP diet

In the chyme collected at the ileal site T and CT activities increased when 2.9% or 0.6% pea ANF concentrates were added to PP diets. More free T and CT were therefore be present when ANFs were added. A reverse tendency was observed at the jejunal site. The reasons for this difference could be (1) the release of active T and CT that were bound to the inhibitors or to proteins in the jejunal chyme or(and) (2) a slower rate of breakdown of the enzymes when

dietary ANF concentrates are present. However protease-PI complexes are known to be very stable. The dissociation of T and CT from PI in the ileum seems therefore hardly possible. On the other hand, a protection of the digestive enzymes from breakdown might have been involved. Undigested dietary proteins in the small intestine may protect enzymes from autodigestion as suggested by Porter and Rolls (1971) and Percival and Schneeman (1979). The addition of 2.9% of ANF concentrate adds PI but also proteins that may have a low digestibility as discussed before (Le Guen *et al.*, 1993*a*). These proteins may therefore provide the proteases with a protection against digestion. However it is unlikely that addition of 0.6% of ANF concentrate would bring sufficient amounts of proteins to protect the enzymes. The presence of stable complexes "proteases - PI" in the gastro-intestinal tract may have acted as resistant proteins protecting the enzymes from breakdown.

The "survival" of enzymes did not seem equivalent for both proteases. More active CT was found at the end of the ileum that was found in the jejunum. As for T, its activity did not change from one site to another. CT would then survive better than T in the ileum.

Proteases in ileal chyme - RP diet

With the raw pea diets, the effects of ANF concentrate on proteases measured in the ileal chyme differed from the effects with the PP diets. With the RPP4 diet, T and CT total activities in the total amount of chyme collected during 5 hours were already significantly higher than with the PP4 and PP4^{++} diets, although the TIA in the RPP4 diet was half that in PP4^{++} diet. Veal calves fed raw pea diets have also a higher ileal flow of active trypsin compared to calves fed a milk diet (Lallès and Toullec, 1991, unpublished). The addition of ANF concentrate to the RPP4 diet did not modify the level of enzyme activities at the ileo-caecal junction. These results indicate therefore that the enzyme concentrations are not directly related to the dietary PI content, which is in agreement with Naim *et al.* (1982).

A secretion of pancreatic proteases higher with the RPP4 diet than with the PP4 diet could be an explanation. However no differences in secretion were observed between a RP diet and a PP diet (Le Guen *et al*, 1993*c*). The undigested dietary pea proteins present in the RP chyme (Le Guen *et al*, 1993*a* & 1993*b*) may have been responsible for the "survival" of proteases to the end of the ileum. In a similar manner, acid-precipitated proteins from soybeans fed to rats resulted in growth depression and in pancreatic enlargement although the TIA of the feed was low (Naim *et al.*, 1982). The carbohydrates may be also partly reponsible for the higher trypsin activity for the RPP4 group than for the PP4 group. The non-starch polysaccharide content was 9.0% on dry matter for the RPP4 diet and only 5.4% for the PP4 diet. The presence of water soluble fibres (pectins for instance) may have also protected the enzymes from degradation in the intestinal content, as suggested by Schneeman (1982). Ikegami *et al.* (1990) also showed that addition of apple pectin to diets fed to rats increased protease

activities in the small intestinal contents, without affecting pancreatic content and secretions. As undigested dietary proteins were already present in the chyme, increasing the TIA of the feed by adding pea ANF concentrate to the RPP4 diet ($RPP4^+$ and $RPP4^{++}$ diets) may not have slowed down the enzyme turnover any more than did the RPP4 diet without pea ANF concentrate.

In conclusion, the present study indicates that levels of pancreatic protease activities in the chyme sampled at the distal end of the small intestine are not indicative for the enzyme activities in the chyme of the proximal digestive tract. It indicates also that the interpretation of enzyme activities in digesta is not obvious as these activities are the result of secretion, breakdown, and adsorption. The relation between these activities and the N apparent ileal digestibility is not evident.

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Chapter VI

EFFECTS OF PEA ANTINUTRITIONAL FACTORS ON PROTEASE ACTIVITY IN PANCREATIC TISSUE AND ITS SECRETIONS IN PIGLET

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EFFECTS OF PEA ANTINUTRITIONAL FACTORS ON PROTEASE ACTIVITY IN PANCREATIC TISSUE AND ITS SECRETIONS IN PIGLET

Diets based on raw pea (RP), pea protein isolate (PP) or both (RP+PP), with or without addition of pea antinutritional factors (ANFs) high in protease inhibitors, were fed to piglets in four experiments. The aim was to investigate the effects of these dietary constituents on protease activity in the pancreas and its secretions. The pancreas were collected from piglets of 10 - 15 kg body weight in experiments 1, 2 and 3. The pancreatic secretions were sampled from piglets of 20 - 25 kg body weight in experiment 4. The pancreas were removed from the piglets sacrificed 3.5 hours after the last meal in experiments 1 and 3, and 14 hours after the last meal in experiment 2. Pancreas weights were unaffected by the diets. Trypsin (EC.3.4.21.4) and chymotrypsin (EC 3.4.21.1) activities in the pancreas of piglets fed exclusively raw pea diet (experiment 1) were lower than those from piglets fed the PP diets. No differences were observed on the protease levels in the pancreatic juice between these two diets (experiment 4). Diets based on RP+PP led to the similar levels of T but higher levels of CT in the pancreas than did PP diets (experiment 3). The addition of pea ANF concentrate to PP diets or RP+PP diets (experiments 2 & 3) affected only the levels of CT activities in the pancreas, and only for the RP+PP diet.

Pancreatic enzymes: Proteases: Trypsin: Chymotrypsin: Pancreas: Pancreatic juice: Peas: Antinutritional factors: Trypsin inhibitors: Piglets

INTRODUCTION

Peas (*Pisum sativum*) are a highly valuable protein source for animals. However their nutritional value is low when they are fed as raw peas to young piglets (Green, 1988; Buraczewska *et al.*, 1989; Huisman *et al.*, 1990*a*; Leterme *et al.*, 1990; Jondreville *et al.*, 1992; Le Guen *et al.*, 1993*a*). The proteinaceous antinutritional factors (ANFs) known in peas have been shown to be partly responsible (Le Guen *et al.*, 1993*a*) through an inhibition of pancreatic proteases in the gastro-intestinal tract by the protease inhibitors (PI) (Le Guen *et*

al., 1993*c*). However an inhibited intestinal proteolysis upon PI inclusion in the diet can coexist with a hypersecretion of digestive enzymes by the pancreas, meaning an inadequacy of the pancreas to counteract the effect of PI (Gertler and Nitsan, 1970). Several studies have shown that soybean PI stimulate pancreatic enzyme secretions, leading to enlarged pancreas, in rats, mice and chicken (Gertler and Nitsan, 1970; Laporte and Trémolières, 1973; Roy and Schneeman, 1981; Temler *et al.*, 1984). The present experiments were designed to investigate the effect of pea PI included in various diets fed to piglets, on the exocrine pancreas, by measuring pancreatic proteases activities in the organ itself and in its secretions.

MATERIALS AND METHODS

1. Design

Four experiments have been carried out on piglets to investigate pancreatic reactions to pea diets.

The objectives of experiments 1, 2 and 3 were to study the variations of potential protease activities in the **pancreas**, upon :

- feeding raw pea (RP) and pea protein isolate (PP) (experiment 1),
- addition of 3% of a pea ANF concentrate (batch a) to a PP diet (experiment 2),
- addition of 0.6% of a pea ANF concentrate (batch b, higher trypsin inhibitor activity than batch a) to a PP diet and to a RP diet (experiment 3).

The objective of experiment 4 was to study the variations of potential protease activities in the **pancreatic secretions** upon feeding raw pea (RP), pea protein isolate (PP) with maize starch, and pea protein isolate (PP) with pea carbohydrate isolate. These pea carbohydrates were isolated during the preparation of the pea protein isolate described by Le Guen *et al.* (1993*a*).

The diet compositions have been previously extensively described (Le Guen *et al.*, 1993*a*). The main components of the diets are reported in table 1, as well as the dietary TIA and lectin contents.

2. Experimental procedures

Experiment 1, 2 & 3:

The piglets were fitted with a PVTC cannula at the terminal end of the ileum for digestibility measurements (Le Guen *et al.* (1993*a*). At the end of the experiments, they were euthanized and bled. After death, their pancreas were removed and quickly frozen after being cleaned and weighed. In experiments 1 and 3, the pancreas were collected 3.5 hours after the morning meal. The animals in experiments 1 and 3 had been on the experimental diets for 25 days and

Table 1. *Feed : brief composition (g/100g), trypsin inhibitor activity and lectin contents*

Experiment ...	1, 4		2		3			
Diets...	*RP2*	*PP2**	*PP3*	*PP3+*	*PP4*	*PP4++*	*RPP4*	*RPP4++*
Whole RP (Fr)	25.0	-	-	-	-	-	-	-
AC RP protein (Fr)	17.8	-	-	-	-	-	-	-
PP (Frijaune)	-	17.9	-	-	-	-	-	-
PP (Finale)	-	-	18.4	16.4	-	-	-	-
Commercial PP	-	-	-	-	15.0	15.0	9.3	9.3
PP (50%Fi+50%Fr)	-	-	-	-	4.0	4.0	2.4	2.4
Raw pea Solara	-	-	-	-	-	-	30.0	30.0
Pea ANFc$^{batch\ a}$	-	-	-	2.9	-	-	-	-
Pea ANFc$^{batch\ b}$	-	-	-	-		-0.6	-	0.6
Maize starch	30.7	52.7	52.2	51.3	51.4	50.8	29.2	28.8
TIA[a]	1.9	0.4	0.1	1.2	0.1	0.8	0.4	1.1
Lectins[b]	1.9	0.5	0.5	2.7	1.5	1.9	1.9	3.4

RP: raw pea; *AC*: air-classified pea protein; *PP*: pea protein isolate. *Fi, Fr*: spring pea Finale and winter pea Frijaune. *Solara*: spring pea; *ANFc*: concentrate of pea ANF, *batch a*: 50% Fr + 50% Fi, *batch b*: 100% Fr
* Experiment 4: the third diet consisted in the PP2 diet in which 27% of the maize starch was replaced by an isolate of Frijaune pea carbohydrates *(PP2+Carb)*
[a] TIA, trypsin inhibitor activity; in mg inhibited trypsin/g feed. [b] Lectins: in mg/g feed.

18 days, respectively. In experiment 2, the pancreas were removed 14 hours after the last meal. The piglets had been 13 days on the experimental diets. The organs were freeze-dried before analysis.

Experiment 4:

Three pigs of 15 kg average initial live weight were housed in metabolism cages adapted for pancreatic juice collection. They were prepared for complete collection and subsequent return of the pancreatic exocrine secretion, by a surgical procedure (Hee *et al.*, 1985). The surgery involved a transection of the duodenum 1 to 2 cm anterior and posterior to the point of entry of the pancreatic duct. A polyethylene catheter was then inserted into this pouch to collect the secretions from the pancreas. The two free ends of the transected duodenum were sutured together and a second catheter was introduced in the duodenum to return the collected secretions. Both catheters were exteriorized through incisions in the flank and secured by an interconnecting piece. The animals were allowed to recover for 2 weeks (2 pigs) and one week (1 pig) because of difference in time of the surgery.

The pigs received each of the three diets in a change-over design where each experimental period lasted 7.5 days. The feed was given as meals in two equal portions at 08.00 and 20.00 hours. Pancreatic juice was collected 12 h a day from 08.00 till 20.00 hours, during the last 3 days of each experimental period. The pancreatic juice flowing freely through the opened catheter into pots kept in ice, was removed every 20 min and weighed. About 10% was sampled and frozen immediately. The remainder was returned to the duodenum by an

automatic pump at a rate similar to the rate of secretion. The juice samples were freeze-dried at the end of each day to avoid any loss of enzyme activity (Makkink *et al.*, 1990; Van Baak and Rietveld, 1991). The dry material of the 3-d collection period was pooled per animal on a weight basis.

The animals weighed about 18 kg during the first period, 22 kg during the second period and 25 kg during the third period.

3. Chemical analyses

A "freeze-drying" dry matter content was determined by freeze-drying the pancreas to constant weight.

Trypsin inhibitor activity was determined using the Kakade method as modified by van Oort *et al.* (1989). Lectin content was measured according to an ELISA technique (Hamer *et al.*, 1989).

Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) activities were determined after activation of the zymogen with enterokinase (EC 3.4.21.9) during 24 h, using a spectrophotometer (Perkin-Elmer 550), and p-toluenesulfonyl-L-arginine methyl ester (TAME) and benzoyl-L-tyrosine ethyl ester (BTEE) as substrates, respectively, according to Bergmeyer (1974). The activities are expressed as units (U), one unit being defined as the amount of enzyme which hydrolyses one μmol substrate per min under the assay conditions.

Total protein in the pancreatic juice was determined by the method of Lowry *et al.* (1951).

4. Calculations and statistical procedures

A one-way analysis of variance was carried out separately for each experiment 1, 2 and 3, using the software package SPSS/PC (Norusis, 1988). The following model was used:

(1) $$Y_{ij} = \mu + D_i + e_{ij}$$

where Y_i = dependent variable, μ = mean, D_i = diet effect and e_i = residual error within the groups of animals, i = 1, 2 for experiments 1 & 2 and i = 1 .. 4 for experiment 3.

Data on pancreatic secretions (experiment 4) were subjected to a two-way analysis of variance according to the following model:

(2) $$Y_{ijkl} = \mu + D_i + A_j + P_k + e_{ijkl}$$

where Y_{ijk} = dependent variable, μ = mean, D_i = diet effect, A_j = animal effect, P_k = period effect, and e_{ijk} = residual error, with i, j and k = 1, 2, 3.

The significance of differences between treatment means was tested by calculating the variances ratio *F* (Snedecor and Cochran, 1980). All statements of significance are based on a probability of less than 0.05.

RESULTS

The protease activities in the pancreatic tissue and secretions were measured after activation of the zymogens. This means that they represent therefore potential activities.

Pancreatic secretions

The animals performed well as indicated by their average daily weight gain of 481 g, for a mean daily feed intake of 835 g.

The amounts of pancreatic fluid and enzymes secreted are shown in Table 2 and Figure 1.

The volume of fluid was higher during the period 6-12h than during 0-6h after feeding and the mean enzyme activities were highest during the first 3h after feeding. As for the daily output of juice, protein and proteases, they were not affected by the dietary treatment. On the average, 700 to 750 g of juice, containing 5 g of proteins, 93 - 110.10^3 units of trypsin activity and 17 - 22.10^3 units of chymotrypsin activities were secreted over 12h.

Table 2. *Secretion of pancreatic fluid and enzymes by 3 pigs of 20-25kg for 12 h, fed diets containing raw peas (RP2) or pea protein isolate (PP2 and PP2+Carb) (experiment 4)*

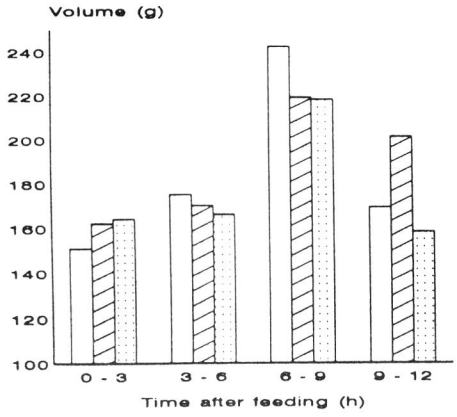
Diet...	*RP2*	*PP2*	*PP2+Carb*	F value	P
Feed intake (g/meal)	380	386	387		
N intake (g/meal)	9.8	10.5	10.9		
Total amount of juice (g)	733.3	753.3	703.3	0.19	NS
Total protein output (g)	5.2	4.9	5.2	0.21	NS
Total activity produced in 12 h (10^3 Units)					
trypsin (T)	93.1	96.3	110.3	0.72	NS
chymotrypsin (CT)	16.6	18.9	22.0	1.54	NS
Ratio T/CT	5.6	5.2	5.4	3.76	NS

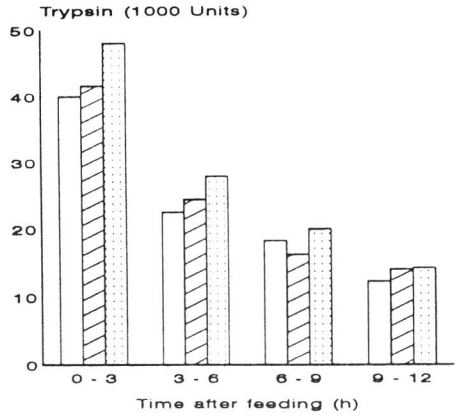
F value: Fisher's value, P: probability of a significant effect of the dietary treatment. NS: not significant ($P > 0.05$).

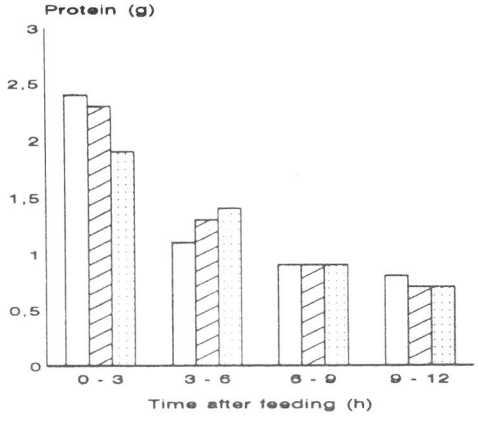
U for trypsin T: amount of enzyme required to hydrolyse one μmol of TAME per min

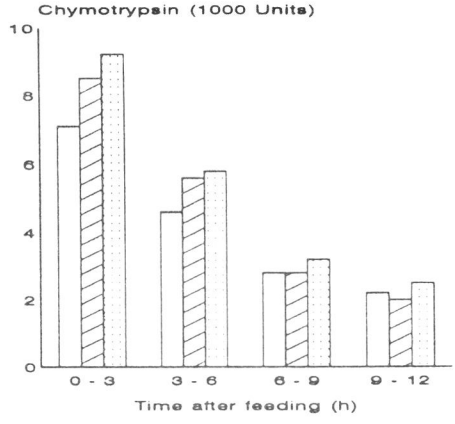
U for chymotrypsin CT: amount of enzyme required to hydrolyse one μmol of BTEE per min

Figure 1. *Pancreatic secretion of fluid and enzymes during 3 hours periods by 3 pigs of 20-25kg for 12 h, fed on diets containing raw peas (RP2 □) or pea protein isolate (PP2 ▨ and PP2+Carb ▨) (Experiment 4)*









Pancreas.

The results are given in table 3.

The piglets of experiment 1 showed a higher live weight than those of the other experiments, as they were kept longer in the experiment. The piglets had received the experimental diets for 25, 13 and 18 days in experiments 1, 2 and 3 respectively.

The pancreas weights, expressed as total or relative to body weight, were not affected by the dietary treatments.

Trypsin (T) and chymotrypsin (CT) activities in the pancreas, expressed as total or relative to pancreas weight, were significantly affected by the protein sources. In experiment 1, the activities were lower ($p < 0.05$) in the pancreas of the piglets fed the high-level raw pea diet (RP2) than in the pancreas of those fed the PP2 diet (specific activity: -37% for T, - 28% for CT; total activity: -54% for T, -45% for CT). In experiment 3, T and CT activities were higher when the piglets were fed the raw pea + pea protein isolate diet (RPP4) than when fed PP4 diet (specific: +37% for T, + 44% for CT; total: +33% for T, +48% for CT).

The addition of 3% of pea ANF concentrate (experiment 2) did not change the T and CT activities in the pancreas. In experiment 3, the addition of 0.6% of pea ANF concentrate to the PP4 diet increased the enzyme activities in the pancreas but not significantly. Added to the RPP4 diet, the ANF concentrate decreased the enzyme activities in the pancreas, significantly only for CT.

The ratios of T/CT were not affected either by the pea protein sources or by the addition of pea ANF concentrate. The ratios were higher in experiment 1 than in experiment 2 and 3. The ratios in experiments 2 and 3 were similar although the levels of activities were somewhat higher in experiment 2 than the levels in experiment 3.

Table 3. *Pancreas weight and protease activity in the pancreatic tissue of piglets fed pea protein diets (experiments 1, 2 and 3) (Mean values with their standard errors)*

Experiment ...	1		2		3			
Diets...	*RP2*	*PP2*	*PP3*	*PP3+*	*PP4*	*PP4++*	*RPP4*	*RPP4++*
n observations	6	6	5	5	6	6	6	5
Feed intake (g/d)	552	548	558	557	475	473	473	480
	(10)	(9)	(4)	(5)	(13)	(9)	(11)	(9)
N intake (g/d)	14.2	14.5	15.6	15.7	12.8	13.2	12.3	12.7
	(.2)	(.2)	(.09)	(.1)	(.4)	(.2)	(.3)	(.3)
Live weight (kg)	18.9	19.8	16.7	16.0	14.8	14.9	14.3	14.3
	(.3)	(.4)	(.3)	(.2)	(.5)	(.4)	(.5)	(.4)
Feeding state	*fed (3.5h p.p.)*		*unfed (14h p.p.)*		*fed (3.5h p.p.)*			
Pancreas weight								
(g)	27.6[a]	36.6[a]	29.0[a]	25.8[a]	25.8[a]	27.2[a]	26.6[a]	24.5[a]
	(4.2)	(2.3)	(1.5)	(2.1)	(1.3)	(1.6)	(1.6)	(1.0)
(g/kg L.W.)	1.43[a]	1.86[a]	1.65[a]	1.62[a]	1.74[a]	1.83[a]	1.86[a]	1.72[a]
	(.22)	(.11)	(.11)	(.13)	(.07)	(.09)	(.07)	(.09)
Specific activity (U/g wet pancreas)								
T	1360[a]	2164[b]	2350[a]	2330[a]	1022[a]	1230[a]	1327[a]	1106[a]
	(97)	(93)	(182)	(209)	(74)	(149)	(89)	(88)
CT	137[a]	189[a]	329[a]	330[a]	146[a]	172[ab]	210[b]	146[a]
	(9)	(23)	(41)	(35)	(7)	(16)	(15)	(19)
Total activity in the pancreas (10^3 unit)								
T	36.2[a]	78.8[b]	68.4[a]	59.5[a]	26.4[a]	33.6[a]	35.1[a]	26.9[a]
	(5.2)	(4.6	(6.9)	(5.4)	(2.5)	(4.7)	(3.1)	(1.7)
CT	3.77[a]	6.80[b]	9.70[a]	8.58[a]	3.77[a]	4.78[ab]	5.58[b]	3.59[a]
	(.63)	(.70)	(1.66)	(1.17)	(.29)	(.65)	(.58)	(.54)
Ratio T/CT	10.1[a]	12.0[a]	7.4[a]	7.2[a]	7.0[a]	7.2[a]	6.5[a]	7.9[a]
	(.9)	(1.0)	(.6)	(.6)	(.4)	(.6)	(.7)	(.7)

LW : live weight, T: trypsin, CT: chymotrypsin, p.p.: post-prandial

Unit : quantity of enzyme needed to hydrolyse one micro-mole of substrate (T.A.M.E for trypsin, B.T.E.E. for chymotrypsin) per minute in the assay conditions.

(Values within experiment and line with different superscripts differ ($p < 0.05$)).

DISCUSSION

Pancreatic secretions

The comparison of literature data on pancreatic juice secretions is complicated as many factors influence the final results (feed intake, diets, methods, bodyweight). The volume of juice is influenced mainly by the type of diet (purified ingredients or not) (Partridge *et al.*, 1982), the feed intake and the levels as well as sources of dietary fibre (Zebrowska, 1985; Zebrowska and Low, 1987).

In the present study, the raw pea diets fed to pigs did not affect the volume of pancreatic juice compared to the PP diets. In the literature, barley-based diet given to pigs as a whole or mixed with raw peas did not affect pancreatic juice output (Buraczewska *et al.*, 1991). On the other hand raw soybean diet compared to heated soybean diet stimulated the secretion of fluid in pigs by 40% (Zebrowska *et al.*, 1985) and by 21% (Corring *et al.*, 1986). The volumes of collected juice are similar to the values reported in the literature for similar diets, e.g. 790 ml secreted in 12 h by pigs weighing 36 kg fed heated soybean meal (Corring *et al.*, 1986), 602 ml secreted by pigs of 35 kg fed a starch-casein diet (Zebrowska *et al.*, 1983), and 650 ml secreted by pigs weighing 48 kg fed a starch-casein diet (Partridge *et al.*, 1982).

Pancreatic protease activities were not affected by the experimental diets, whether they contained a high or a low TIA. This is in agreement with Buraczewska *et al.* (1991). In the studies of Zebrowska *et al.* (1985) and Corring *et al.* (1986) pigs fed soybean diets were used for pancreatic juice collection. The total outputs of protein and enzyme in these studies were also unchanged whether the soybean TIA was eliminated by heat treatment or not. The protease activities measured in the pancreatic juice in the present study were comparable with those measured by Partridge *et al.* (1982), as the diets were semi-synthetic and the methods to assess the activities were the same. Their results were similar to the present ones: 90.10^3 U for trypsin and 26.10^3 U for chymotrypsin for a starch-casein diet.

From experiment 4, it can be concluded that, in pigs, raw pea diets of a high TIA do not stimulate the secretion of juice and proteases from the pancreas, compared to a PP diet of a low TIA.

The pattern of secretion of pancreatic fluid and proteases during the time course was similar to that observed by Ternouth *et al.* (1974, 1975) who collected pancreatic secretions from calves fed different types of diets. Similarly Zebrowska *et al.* (1985) showed similar patterns of pancreatic flow for pigs.

Pancreas

Pancreas weights were not affected by any of the experimental diets, as has been observed in many studies using pigs fed diets containing PI (Yen *et al.*, 1977; Struthers *et al.*, 1983;

Huisman *et al.*, 1990*b*). In contrast, similar studies reported enlarged pancreas when using chicken (Garlich and Neisheim, 1966; Gertler and Nitsan, 1970) and rats (Khayambashi and Lyman, 1966; Roy and Schneeman, 1981).

In the present study, the measurements of enzyme activities were carried out in pancreas removed from piglets in a fed state or in a fasted state. The effects of diets containing PI on the pancreas can differ according to the feeding state of the rats. Fasting can rapidly smooth the differences between pancreatic enzyme levels (Kai *et al.*, 1984; Crass *et al.*, 1987). In experiment 2, where the piglets were in a fasted state when sacrificed, the protease activities in the pancreas were higher than when the animals were fed (experiment 3). This is in agreement with Kakade *et al.* (1973) and Crass *et al.* (1987).

The levels of protease activity in the pancreas removed from unfasted piglets fed two similar diets (PP2 and PP4) are different between experiments 1 and 3. This could be explained by the differences in live weight of the piglets and in the duration of experimental feeding duration, according to Gorrill and Thomas (1967). The amount of proteases in the pancreas of a fed animal at a given time after feeding may also depend on the secretion rate for this animal.

Continuous feeding of a diet based on only raw peas as the protein source (RP2) instead of based on PP (PP2) decreased pancreatic T and CT activities in the pancreas. Such an effect was also observed in the pancreas of unfasted piglets (Yen *et al.*, 1977) and of unfasted young guinea-pigs (Hasdai *et al.*, 1989), fed raw soybeans (RSB) *vs.* heated soybeans (HSB). Struthers *et al.* (1983) also found lower pancreatic trypsin and chymotrypsin in fasted pigs fed RSB diet *vs.* HSB or casein diet. Gorrill and Thomas (1967) and Guilloteau *et al.* (1986) also found a decreased activity in the pancreas of calves fed soyflour diets compared to calves fed milk diet.

If the PI were implicated in the effect of the RP2 diet on the pancreas, the reduced protease activities in the pancreas could have been the result of a hypersecretion of proteases in the intestinal lumen according to the feedback mechanism (Birk, 1989; Gallaher and Schneeman, 1986). However the effect of the RP2 diet could hardly be accounted for the PI for the following reasons. First, the addition of pea ANF concentrate to the RP+PP or PP diets (RPP4 and PP4) did not affect significantly pancreatic trypsin activities. However it should be noted that these ANF additions never led to the same TIA as in the RP2 diet. Secondly, experiment 4 also showed that the secretion of proteases was similar whether the diets were RP2 (TIA = 1.9 mg trypsin inhibited/g) or PP2 (TIA = 0.1 mg trypsin inhibited/g). In addition, in a previous study, Le Guen *et al.* (1993*c*) have shown that pea ANF concentrate addition to a PP diet decreased protease activities in the upper small intestine. What might possibly have been the first limiting factor for the regulation mechanism specific to PI feeding to occur with the RP2 diet is the nutritional quality of the protein source, in relation to the

rapid protein turnover in the pancreas (Simon *et al.*, 1978). The RP2 diet have been shown to have a low ileal apparent digestibility for N and five essential amino acids (Le Guen *et al.*, 1993*a, b*). The AA supply may have not met the requirement of the pancreas to secrete more proteases and to maintain an adequate synthesis in the tissue. The importance of protein quality on pancreatic physiology has been reported by Green and Nasset (1983) and by Valette *et al.* (1992). According to Malfertheiner *et al.* (1988), the pancreas would not respond to a cholecystokinin stimulation in case of a dietary protein deficiency or a poor quality dietary protein.

The ANF concentrate added to the highly digestible PP diets (PP3 and PP4 diets) did not affect the enzyme activities in the pancreas, and decreased the protease activities in the upper small intestine (Le Guen *et al.*, 1993*c*). It seems likely that with PP diets, the supply in AA for the pancreas to maintain protease synthesis and the supply of proteases in the small intestine would be sufficient, even when part of the enzymes are inhibited by dietary PI.

CONCLUSION

The diets based on raw peas, when fed continuously to piglets, led to reduced proteases activity in the pancreas during the course of digestion. An hypersecretion would not be the reason for this decrease as the same levels of protease activity were found in the pancreatic juice of piglets fed these two proteins sources. Pea ANF concentrates, including PI, added to PP or mixed RP+PP diets did not modify protease activities in the pancreas. The present study also showed that protease content in the pancreas collected from unfasted animals does not reflect the post-prandial secretion.

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Chapter VII

GENERAL DISCUSSION

GENERAL DISCUSSION

1. INTRODUCTION

White-flowered peas (*Pisum sativum*) are valuable energy and protein sources for both human and animal. However, their nutritional value for monogastric animals is lower than the value of toasted soybean meal, and is also highly variable as indicated by energy and nitrogen digestibility (Gdala *et al.*, 1992; Perez and Bourdon, 1992). The chemical composition of commercial peas is highly variable (Guéguen and Barbot, 1988). Spring white-flowered peas, mostly grown in France, contain 20 to 29% crude protein, 42 to 58% starch, 4 to 8% crude fibre (relative to dry matter content). Within the protein fraction, the proportions of the different categories of proteins also vary according to phenotype and genotype (Baniel *et al.*, 1992; Guéguen and Barbot, 1988).

Considering the increased production of peas in France (3.2 MT, in 1992) and EC (3.9 MT, in 1992) and its utilisation as a substitute for imported protein-rich feedstuffs in animal feed, it is essential to identify the factors responsible for the unsatisfactory and variable nutritional value of peas. This knowledge can be useful to feed compounders to select batches of peas that will allow optimum animal growth performance or to apply biotechnological treatments to improve peas. In a longer term, the informations can be used by plant breeders to select varieties of constant and high nutritional value.

The pea protein is composed of two main fractions, namely globulins (60%) and albumins (25%). The remaining (15%) exists in the form of insoluble proteins. The globulins are the major storage proteins, characterized by high molecular weights (> 150 kD) and a deficiency in sulphur amino acids and in tryptophan. The albumins are biologically active proteins, characterized by molecular weights lower than 25 kD and a high content in sulphur amino acids, especially cystine. Some albumins have antinutritional properties, and therefore called antinutritional factors (ANFs). The main protein ANFs in peas have the property to form complexes with trypsin and chymotrypsin enzymes, and therefore generally referred to as protease inhibitors (PI), and also to bind to carbohydrates components, and generally referred to as the lectins.

In the present study, raw pea proteins and isolated pea proteins were evaluated for their ileal digestibility and their physiological effects on pancreatic enzymes in piglets. The amino acids and the N apparent ileal digestibilities of raw peas were first determined as the base to the research. The main components of peas and the pea ANFs were isolated. Their responsibilities

in raw pea digestibility were evaluated. These aspects as well as the implication of other factors as accessibility or antigenicity are discussed here. Since the literature indicates possible effects of PI on exocrine pancreas, the influences of pea ANFs on digestive process were evaluated through determinations of protease activity in pancreatic tissue and in intestinal digesta. A general scheme on digestive processes in relation to PI is presented here.

2. NITROGEN AND AMINO ACID DIGESTIBILITY OF RAW PEA DIETS

2.1. Target animal

The nutritional studies focussed on pigs as the incorporation of peas in pig diets represent an important opening. Piglets were used as a model for these studies because of their high sensitivity to dietary variations compared to the older pigs. Rats or mice were not used as they respond differently to pea feeding compared to piglets (Huisman *et al.*, 1990*a*). Chickens, also an important consumer of peas among farm animals, were not the best model for ANF research because they are less sensitive to pea diets than piglets (Huisman *et al.*, 1990*a*).

2.2. Digestibility determination

For optimum growth performance of young monogastric farm animals, their nutritional requirements need to be satisfied. The chemical composition of the diets is not a satisfactory indication of performance as nutrients present in a feedstuff, e.g. amino acids, carbohydrates, lipids, vitamins, minerals, are not completely available to the animals. The digestibility measurements of the dietary nutrients is an approach well recognised to evaluate the availability of nutrients for an animal species. The ileal instead of the fecal digestibility measurement was chosen to evaluate pea diets as the ileal method provides a more reliable estimation of the nutrients potentially available, particularly of amino acids (Sauer and Ozimek, 1986; Just *et al.*, 1981). Moreover, ileal digestibility is a more sensitive method than fecal digestibility (Sauer and Ozimek, 1986; van Weerden *et al.*, 1985).

2.3. Digestibility of raw peas

Ileal digestibility experiments were carried out in piglets with diets based exclusively on raw peas as the protein source. The raw peas used in the diets were spring varieties (Finale, Solara) which were genetically similar and low in trypsin inhibitor activity (TIA), and a winter variety (Frijaune) which was high in TIA.

The digestibility results presented in chapter III show that diets based on raw peas as the sole protein source were not well digested in the small intestine of piglets. The N apparent ileal

digestibility averaged 69 - 70% regardless whether the raw peas contained a high or a low TIA. A similar N digestibility for both pea diets differing in their TIA, indicates that TIA may not be the main factor responsible for the low digestibility of raw peas. It is also possible that from a specific TIA threshold level onwards, the N digestibility is not affected any further. The DM digestibility averaged 74% and 77% for Finale and Frijaune diets, respectively. Four essential amino acids (MET, CYS, THR, TRP) showed, in both varieties, low apparent ileal digestibility coefficients ranging from 34% to 63%. This explains why the mean daily weight gain of the piglets fed the RP diets was lower than the control group (240 vs. 320 g/d). The amounts of feces excreted by the piglets fed raw peas were 48% higher compared to control groups. Improving the digestibility of raw peas appears worthwhile in relation to environmental concern.

3. FACTORS EFFECTING THE NITROGEN DIGESTIBILITY OF RAW PEAS

To understand the unsatisfactory apparent ileal digestibility of raw peas in young pigs, other digestibility experiments were carried out using the following pea fractions:

- pea protein isolates (Finale and Frijaune variety, 90% crude protein) consisting mainly of globulin proteins, devoid of pea carbohydrates and ANFs. These were produced by acid precipitation

- pea ANF concentrates (70% crude protein): pea protein, mainly albumins, having antinutritional properties (PI and lectins). These were produced by ultrafiltration of the whey fraction after precipitation of the globulins.

- pea carbohydrate isolates prepared from pea flour after solubilisation of the proteins.

3.1. Pea proteins

3.1.1. Non-ANF pea proteins

Pea protein isolates (PP) devoid of pea carbohydrates and pea ANFs were used to investigate whether the pea proteins were the cause of the low N apparent ileal digestibility of raw peas in piglets. It should be noted that the proteins found in these isolates differ qualitatively from those found in the raw pea. In raw peas, the globulins represent about 60% of the crude protein. However, in the pea protein isolate, the globulins represent about 80% of the crude protein as the other main proteins e.g. the albumins - are present mainly in the whey fraction and not the precipitate.

The diets based on PP showed high N apparent ileal digestibility coefficients (84 - 85%) for Finale and Frijaune varieties (Chapter III), about 16 units higher than N digestibility of the RP

diets. The AA apparent digestibility was also much higher in PP diets than in RP diets, particularly for TRP and MET amino acids (Chapter IV). However the apparent ileal digestibility coefficients of MET, CYS, THR and TRP ranging from 61% to 75% were again lower than N apparent ileal digestibility in PP diets.

An attempt was made to determine the origin of the amino acids (both exogenous and endogenous) present in the ileal chyme collected from piglets fed diets based on raw pea (RP) or pea protein isolate (PP) devoid of ANFs and pea carbohydrates (Chapter IV). From the ileal digestibility determinations, it was found that 4.3 g N/day and 2.3 g N/day flowed from the small intestine into the large intestine for the RP and PP diets, respectively. By means of multiple regression calculations described in chapter IV, it appears that for the RP diets, 20 to 25% of the N leaving the small intestine would be of dietary origin (0.9 - 1.1 dietary N g/day) and the rest of endogenous origin (3.2 - 3.4 endogenous N g/day) (Chapter IV). This indicates that the low apparent N digestibility of raw pea diets in young pigs is mainly due to the endogenous N. The calculations showed that the undigested dietary N in the ileal chyme would be albumin proteins (Chapter IV). For the PP diets, the calculations showed that all the N leaving the small intestine would be of endogenous origin (2.3 g/day) (Chapter IV). This would mean that the true digestibility of pea protein isolate would thus reach about 100%. As PP contains mainly globulins as explained before, it would mean that pea globulins would have a high true digestibility. This observation, plus the facts that the undigested dietary N in the RP digesta would be albumin proteins and that the true N digestibility of RP diets measured according to the ^{15}N technique (Huisman *et al.*, 1992) would equal 92 to 95%, indicate that pea albumins would be low digestible.

3.1.2. Proteins containing antinutritional properties

During the preparation of the pea protein isolates, the major part of the albumin proteins remained in the whey fraction. Because protease inhibitors and lectins are of albumin type, the major part of these ANFs was found in the whey. After ultrafiltration and air-drying, the whey fraction gave the so-called ANF concentrate.

A first ANF concentrate was prepared (batch a) and added to PP diet at a level of 2.9% (Chapter III). It was observed that the N apparent ileal digestibility of the PP diet decreased by 7 units and the daily weight gain of the piglets was reduced by 17%. As discussed in chapter III, the negative effect of the ANF concentrate could have been due to a low digestibility of the albumin proteins as well as to their antinutritional properties.

No data are available concerning the *in vivo* digestibility of pea albumins. However, as discussed previously, there are indications that they might not be well digested. Their high CYS content indicates the presence of a large number of disulfide bonds, known to be resistant to hydrolysis by enzymes. In addition, Aubry and Boucrot (1986) observed that

radiolabelled pea lectins were less hydrolysed than pea globulins in the gastro-intestinal tract of rats. Gatehouse *et al.* (1985) observed the resistance of pea albumins to hydrolysis during germination of pea seeds.

The observations mentioned in the previous paragraph support the hypothesis that pea albumins might have a low digestibility. Moreover, the addition to a PP diet, of 0.6% of a pea ANF concentrate (batch *b*) of a higher TIA that the one added at a level of 2.9% (batch *a*), did not affect the N ileal digestibility in spite of the increased TIA of the diet. This indicates that the lowered digestibility of the diet PP+3% ANF may be linked to the low digestibility of the proteins of the ANF concentrate. However this does not discard the possible implication of the protease inhibitors in the low nutritional value of peas for piglets. Indeed, it was observed that addition of 0.6% of pea ANF concentrate (batch *b*) decreased the N apparent ileal digestibility when the diet was based on RP+PP although it did not when the diet was based on PP only (Chapter III). This difference may probably be accounted for the TIA of the ANF concentrate and not the albumin proteins themselves.

Nevertheless, albumins and PI do not explain entirely the difference in digestibility between RP and PP. This is consistent with the linear correlation coefficient of -0.70 between TIA of pea diets and N digestibility in pigs and piglets (the present study; Perez and Bourdon, 1992), indicating that 50% of the variation in N digestibility of pea diets is due to other factors.

3.1.3. Antigenicity of proteins

Another aspect that may be relevant to explain the low apparent ileal digestibility of raw pea N in piglets is the antigenicity of pea proteins. In a pilot experiment, piglets fed the RP diets described in the present study developed immunoglobulins (IgG) against pea proteins (Le Guen *et al.*, 1991). It means that pea polypeptides had been absorbed from the intestine lumen into the blood. They escaped complete proteolytic digestion in the intestine, so they were large enough to stimulate the immune system. The large proportion of endogenous N in the ileal chyme from piglets fed raw peas (paragraph 3.1.1) could be due partly to the immune reaction through an increase of mucus production and desquamation of the epithelium in the small intestine (Lallès, 1991). Bacterial population may also be increased due to changes in gastro-intestinal motility and in gut permeability.

In a recent experiment (Le Guen & Tolman, unpublished, 1992) it was found that, among piglets fed a PP diet or a RP diet, some of them developed antibodies against pea proteins, of a similar magnitude for both diets. The apparent ileal digestibility of N and the performance of the animals were much higher for the PP group than for the RP group. It was also observed that, in the RP group, the piglets that did not form antibodies did not have a higher N apparent ileal digestibility than had the piglets that formed antibodies. From these results, it

seems that the antigenicity of pea proteins is not partly responsible for the low digestive utilisation of N. However this aspect need to be studied in more details.

3.2. Carbohydrates

The implications of carbohydrates in the digestion process were studied using isolated carbohydrate fractions. Three control diets based on three protein sources were formulated. For each of them, part of the maize starch was replaced by pea carbohydrate isolate. These three test diets were formulated to match the carbohydrate composition of the RP diet. The experiment was reported in details by Huisman *et al.* (1990*b*). The main results are given in table 1.

Table 1. *Mean dry matter content of ileal chyme and apparent ileal digestibility of dry matter (DM) and crude protein (CP) of diets fed to piglets 10 - 15 kg liveweight (Huisman et al., 1990b)*

Protein source...	Casein + Fish meal		PP Finale		PP Frijaune	
Pea carbohydrates...	-	+	-	+	-	+
Observations	4	4	4	3	3	3
Ileal chyme:						
g wet/12h	244	409	323	424	275	347
	(10)	(40)	(12)	(72)	(32)	(55)
g dry/12h	33	39	39	46	38	45
	(2)	(7)	(2)	(6)	(3)	(1)
% DM	13.6^{a}	11.1^{b}	12.1^{ab}	10.9^{b}	14.1^{a}	13.2^{b}
	(1.2)	(1.2)	(.4)	(.5)	(2.0)	(1.8)
Ileal apparent digestibility (%)						
DM	86.1^{a}	82.7^{bcde}	84.1^{ce}	80.8^{d}	84.5^{ae}	81.9^{bcd}
	(1.2)	(1.0)	(1.1)	(1.8)	(1.1)	(.3)
CP	87.1^{a}	84.8^{a}	88.2^{a}	86.7^{a}	85.8^{a}	86.0^{a}
	(2.1)	(2.3)	(2.4)	(1.7)	(1.9)	(1.9)

Mean and standard deviation. PP: pea protein isolate. Values within lines with different superscripts differ ($p<0.05$).

It was found that incorporation of isolated pea carbohydrates in a diet fed to piglets increased the amounts of ileal chyme. The percentage of water in the small intestinal chyme was increased for the test groups (Table 1). The apparent ileal digestibility of the dry matter was lower when the pea carbohydrates were present in the diets. Apparent ileal digestibility of N was not affected by the incorporation of pea carbohydrates irrespective of the protein source. The latter aspect is discussed in the following paragraphs.

3.2.1. Non Starch Polysaccharides

Including pea carbohydrates in diets increased the amounts of chyme and decreased the DM content of the chyme (Table 1). These modifications may have been due to water-soluble NSP, especially the large molecules, like pectic substances, known for the ability to retain water molecules and to increase intestinal viscosity (Classen and Bedford, 1991). Moreover, the amylose chains, making up 35% of pea starch, have the property of forming compact gels of a low α-amylolysis susceptibility (Colonna *et al.*, 1992).

Reports in the literature indicate that water soluble NSP of a high viscosity potential may be expected to decrease the N apparent ileal digestibility because of their possible effects on enzyme diffusion in the intestinal content, on enzyme activities (Ikegami *et al.*, 1990; Schneeman, 1982), on gutwall morphology (Paulini *et al.*, 1987), on microbial activity (Costa *et al.*, 1989). However, in the reported experiment (Table 1), the N digestibility was not affected. The ileal digestibility of the protein sources used in these diets was high and may therefore not be influenced by the incorporation of pea carbohydrates. Moreover, the incorporation of pea proteins and pea carbohydrates in a diet may not be equivalent by means of isolated fractions and by means of raw pea meal. Isolated components are available to hydrolysis already in the stomach. In raw pea, however, they can be hydrolysed when they are released from the cells. The effects of NSP may be different according to the site of hydrolysis of the proteins.

3.2.2 Cell walls

Isolation of protein and the carbohydrate fractions from raw peas to evaluate their effects on digestibility leaves out one factor that may play also an important role in the digestibility: the accessibility to the endoplasmic content of the endosperm cell.

An isolation or purification process consists in separating the desired components from the rest. The seeds should be ground very finely for the content of the cells to become accessible (Guéguen, 1983). In addition, the links between the components have to be broken. However, when raw pea flour is incorporated in a diet, the grinding of the seeds is not as fine as for a purification process. Therefore the pea cells are less exposed to hydrolysis by enzymes. The content of the endosperm cells will be digested by the animal if the enzymes can have access to it, therefore if the cell walls can be partly broken.

In young chickens fed raw peas, a linear correlation ($r = 0.886$, $p < 0.001$) was found between the digestibility of starch and the digestibility of crude protein, indicating that one factor affected both nutrients in a similar manner (Carré *et al.*, 1991). The presence of high amounts of undigested pea starch in the large particles of the excreta suggested that the non-accessibility to the cellular content for enzymes may be responsible (Carré *et al.*, 1991).

4. PROTEASE INHIBITORS: EFFECTS ON PANCREAS

From experiments mainly with soybeans fed to rats , it has been generally explained that dietary protease inhibitors (PI) entering the intestinal lumen inactivate pancreatic trypsin and chymotrypsin, thereby preventing normal feedback regulation and stimulating pancreatic protease secretion (Liener and Kakade, 1980). However this general mechanism is submitted to several factors that may modify it as reviewed in chapter II. Several aspects concerning the pancreas were studied in the *in vivo* experiments. Trypsin and chymotrypsin activities were measured in the pancreatic tissue, pancreatic secretions, jejunal chyme and the ileal chyme collected from piglets fed raw pea diets or PP diets, with or without addition of pea ANF concentrates. The results are summarized in table 2.

Table 2. *Trypsin activity measured at different sites of the small intestine in piglets fed raw pea (RP) or pea protein isolate (PP) diets with or without ANF concentrate, expressed relative to the activity for the (PP,-) diet.*

Protein source...	RP	PP				RP + PP	
Pea ANF concentrate...	-	-	-	+ 3%[a]	+.6%[b]	-	+ .6%
TIA of feed	1.9	0.4[a]	0.1[b]	1.2[a]	0.8[b]	0.4	1.1
DC_N[c] (%)	70	86	85	79	84	81	78
pancreas	63	**100**	**100**	100	100	100	100
pancreatic secretions	100	**100**	-	-	-	-	-
jejunal chyme	-	-	**100**	-	65	-	-
ileal chyme	-	**100**	**100**	209	206	180	184

TIA: trypsin inhibitor activity, mg inhibited trypsin/g of feed
[a]: experiment "a" with addition of 2.9% of pea ANF concentrate (batch a)
[b]: experiment "b" with addition of 0.6% of pea ANF concentrate (batch b, higher TIA than batch a)
[c]: apparent ileal digestibility coefficient for N of the diet

Each site in the gastro-intestinal tract where measurements have been made has different influences on pancreatic protease activities. The possible reasons for this are discussed in the following sections.

4.1. Pancreatic tissue and secretions

It was observed that the secretion of enzymes for the RP and the PP diets was similar (Chapter VI). Whether the TIA of the feed was high (RP diet) or low (PP diet), the secretion was not affected. Therefore, when feeding the RP diet, the high amount of endogenous N leaving the small intestine would not originate from a higher pancreatic secretion.

The pancreas of the RP group contained less protease activity than the pancreas of the PP group (Chapter VI). As this was measured in non-fasted piglets, the activity was the result of secretion and biosynthesis of zymogens in the organ. As the exports of proteases via the pancreatic secretions were the same for RP and PP groups, it would mean that the piglets fed the RP diet would have been unable to maintain in their pancreas a biosynthesis simultaneously to the secretion, in contrast to the piglets fed the PP diet (Chapter V). This could be linked to the low digestibility of some essential AA of the RP diet. The pancreas may not have been supplied with sufficient amounts of the correct amino acids to synthesize and secrete more enzymes.

These results indicate also that enzyme activities measured in the pancreatic tissue from non-fasted animals would not reflect enzyme activity secreted in the intestinal lumen.

The addition of pea ANF concentrate to the diets did not affect the levels of enzyme activities in the pancreas. However no conclusions can be drawn for the effects of ANF concentrate on enzyme secretion.

4.2. Jejunal chyme

The addition of a concentrate of pea ANFs to the PP diet reduced the protease activities in the jejunal chyme (Chapter V). The activity measured is the result from the activation of the secreted zymogens and from the inhibition by the PI. On one hand, a decreased activity may indicate that no stimulation of the pancreas occurred when PI were added to the PP diet. This is perhaps due to the high N ileal digestibility of the pea protein isolate. Enough active enzymes could still be present in the upper intestine after partial inhibition by PI. The threshold level of active enzymes under which the negative feedback mechanism is suppressed and the pancreas is stimulated, would therefore not be reached.

However an increased secretion of enzyme may have occurred. Indeed, the PI may have inhibited the extra-secreted enzymes and part of the normally secreted enzymes. This would also result in a decreased activity.

4.3. Ileal chyme

The measurements done in the chyme sampled at the ileo-caecal level indicated an increase of protease activities when ANF concentrates were added to PP diet or when raw peas replaced part of the PP (RP+PP diet) (Chapter V). The addition of ANF concentrate to the latter diet did not affect the enzyme activities in the ileal chyme. The ANF effects as observed in the present studies differ therefore according to the feed composition.

For the PP diets, the increased activity of proteases upon addition of ANF concentrate could be the result of an increased pancreatic secretion of protease due to PI. However, in the jejunal chyme, the activities were lower upon addition of ANF concentrate. It would mean that the surplus of proteolytic enzymes secreted by the pancreas were complexed with the PI in the jejunal chyme and became free in the ileum. It would mean that the extra secretion could be shown in the ileal chyme and not in the jejunal chyme, under the experimental condition.
However it is possible that no stimulation of pancreatic secretions occurred upon addition of ANF concentrate (see 4.2). The higher enzyme activity at the end of the small intestine could then be due to a lower breakdown of the enzymes caused by the undigested ANF proteins or the PI-protease complexes (discussion Chapter V).
Although the enzyme activities were higher due to addition of ANF concentrate, the N apparent ileal digestibility remained unchanged when 0.6% of ANF concentrate were added (Table 2). The relation between both parameters is therefore not apparent.

As for the RP+PP group, the enzyme activity measured in the ileal chyme was higher than that for the PP group. However the RP+PP diet had a low TIA (Table 2). It is likely that the pancreas was not stimulated to secrete more proteases as similar pancreatic secretions were found for the RP and PP diet (Table 2). The higher enzyme activity was probably related to the proteins and to some specific carbohydrates rather than the PI. The presence of undigested pea proteins and water soluble polysaccharide fraction present in pea may have protected the proteases from degradation in the intestine (discussion Chapter IV).
The addition of ANF concentrate to the RP+PP diet did not alter the level of enzyme activity at the ileo-caecal level. If the pancreas secreted more proteases, it could mean that the extra proteases remained bound to the PI until the end of the small intestine. This could be the reason for the decreased N apparent ileal digestibility upon addition of ANF concentrate.

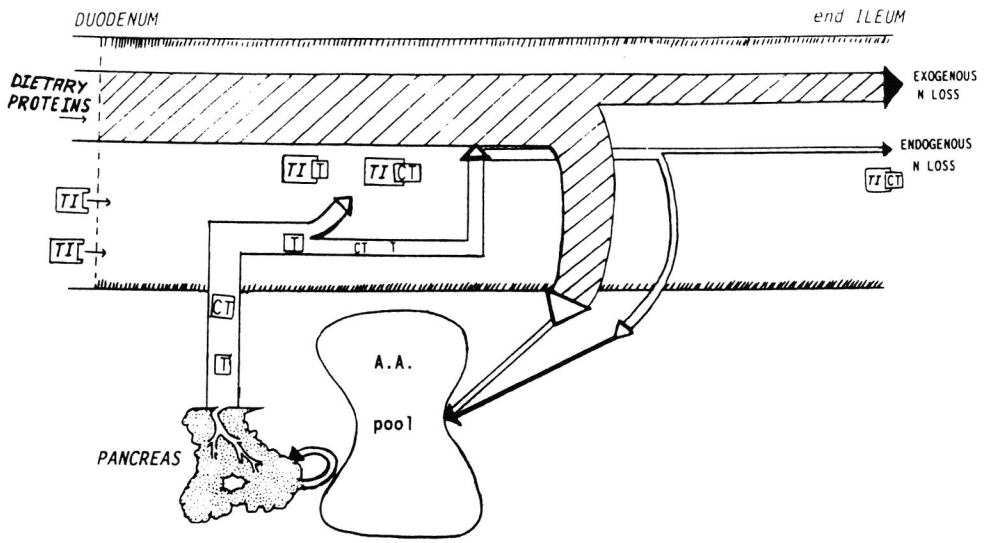
These results are difficult to extrapolate to the exclusively RP diet. It is possible that the effects of an addition of pea ANF concentrate to a PP or a RP+PP diet might be different to the effects of ANF naturally present in raw peas. The accessibility of the pancreatic enzymes to the PI may be better and quicker when the PI are in a concentrated form rather than in native seeds. The effects during the course of digestion might be different depending on the form of the PI. It is therefore not clear whether the high amount of endogenous N found in the ileal chyme of the piglets fed the RP diet was related to a higher secretion of pancreatic proteases or other endogenous sources such as mucus proteins, or to a the lower breakdown and absorption of enzymatic N.

4.4. General concept for mode of action of PI

An attempt was made to schematize the mode of action and the effects of pea PI on the apparent ileal digestibility of proteins according to the nutritional quality of the dietary proteins.

A basal scheme is presented in Figure 1.

Figure 1. Scheme representing the bases of the digestive processes when protease inhibitors enter the small intestine of monogastric animals



The dietary proteins entering the small intestine undergo hydrolysis by protease. The pancreas, while making pro-enzymes, secretes part of these proteases (trypsin T, chymotrypsin CT) in the small intestine. Part of the dietary proteins may escape digestion and leave the small intestine. This constitute what is called the exogenous N losses. Its measurement, by ^{15}N technique for instance, results in the determination of the true nitrogen digestibility. The digested proteins provide the amino acids (AA) pool of the body with dietary AA. The N from digestive secretions of the small intestine is reabsorbed (about 75%) and the rest (about 25%) leaves the small intestine (Souffrant, 1991). The latter fraction constitutes the endogenous N losses. The measurement of N in the digesta for apparent ileal digestibility determination include the endogenous and the exogenous N.

When the active dietary PI enter the small intestine, they bind to T and CT. Part of these complexes "protease - PI" may leave the small intestine in that form and therefore decrease the apparent N digestibility.

Different situations could be encountered upon the inactivation of pancreatic proteases.
The amount of active T and CT, after partial binding to PI, can be lower than the demand for hydrolysis of the dietary proteins. The pancreas would then be stimulated via CCK hormone (Chapter II) to secrete more proteases. Two possibilities may be encountered at this level. The dietary proteins supply enough and adequate AA for the synthesis of pancreatic enzymes. The pancreas can respond to the stimulation by making and secreting more proteases. The dietary proteins could thus be digested as normal. However, as about 25% of the N secreted in the small intestine is not reabsorbed, the endogenous losses increase when the pancreatic secretions of enzymes increase. The result is a reduction of N apparent ileal digestibility via the endogenous component. On the other hand, if the dietary proteins have a low true digestibility for instance, they may not provide the AA pool with the right AA for the synthesis of more proteases. The pancreas can not therefore secrete more enzymes. The consequence could be an increase of exogenous N losses. These non-digested proteins could also affect the breakdown and reabsorption of proteases in the small intestine, and therefore increase the amount of endogenous N. In both case the apparent N digestibility is reduced.
On the other hand, the level of active T and CT after partial binding to PI can still be high enough to hydrolyse the dietary proteins. The pancreas would not be stimulated to produce more enzymes. The PI would therefore not affect the N apparent ileal digestibility.

5. PRACTICAL AND SCIENTIFIC IMPLICATIONS

The research work that has been carried out over the last 5 years on peas has been very useful in understanding the digestive utilisation of raw peas by monogastric animals. The N apparent ileal digestibility of raw pea fed to piglets can be low. Some essential amino acids (MET, CYS, THR, TRP) can have an apparent ileal digestibility lower than that of N. The formulation of feed including peas should therefore take into account the low digestibility of these amino acids in order for the feed to support optimum growth. However one of the priorities is to identify the factor(s) responsible for the unsatisfactory results, and then to study improvement of the product by technology or genetic selection. The incorporation of raw peas in diets could then increase and the formulation security margins decrease, without affecting growth performance and without loading the environment with N excretion.
From the results of the thesis, it is clear that there is not only one factor involved in the low digestive utilisation of raw peas by piglets.
The importance of pea protease inhibitors had not been shown clearly previously. Many studies concluded, from experiments comparing raw peas with extruded peas, that inactivation of protease inhibitors as was done through extrusion processes for instance, improved animal

performance, without considering all the modifications induced by such a treatment on the other nutrients. It was shown in the present experiments that the protease inhibitors contribute to the poor digestive utilisation of pea N by piglets. But the extent of this contribution could not be evaluated because the albumin proteins responsible for the trypsin inhibitor activity might themselves have a low digestibility. There are insufficient informations on the digestibility of pea albumins. Research programs aiming at improving the amino acid profile of pea via genetic manipulations on the albumin proteins should first determine the true digestibility of pea albumins. Chemical modification through disulfide interchange (Friedman and Gumbmann, 1986) could on the other hand perhaps improve the digestibility of pea albumins and at the same time inactivate the PI. Pea globulins would on the other hand be highly digestible.

Pea carbohydrate isolates did not affect N digestibility. Nevertheless, they decreased the DM content of the intestinal content and the DM digestibility. In that respect, the importance of the water soluble NSP such as pectic substances should be evaluated. Special attention should be paid also to the endosperm cell walls with regard to nutrient accessibility. Indeed, the isolation process eliminated from the study the structure of the cotyledon cells. As this may be an important element of the nutritional value, technological treatments based only on heat would not be sufficient to improve the value of raw peas. It may be necessary to apply mechanical forces.

After identification of the major factors responsible for the low digestibility of peas, specific *in vitro* techniques should be set up to characterize pea batches. Biotechnological treatments could also be developed to improve the product.

The estimation of the proportions of exogenous and endogenous protein in the ileal chyme of the piglets fed the RP diet was based on calculations from amino acid profiles using a mathematical model. The method was shown to give realistic results. Most of the amino acids present in the RP digesta were of endogenous origin in agreement with an ^{15}N experiment using the same RP diet (Huisman *et al.*, 1992). Compared to the ^{15}N dilution technique, this mathematical method is much more simple.

The origin of this endogenous N could have been of pancreatic origin in relation to the intake of protease inhibitors. However it was shown that the relation between the apparent ileal digestibility of N and the protease activities at different levels of the gastro-intestinal tract is not obvious. In addition, the relation between protease activity in the pancreatic secretions and protease activity in the intestinal chyme remains to be determined. When protease inhibitors are ingested by the animal, it should be reminded that the activities measured in the intestinal content are the result of secretion, plus inhibition by PI, plus breakdown of enzymes. As for

pancreatic tissue, its protease activity can not be used to investigate the short-term effect of a diet on pancreatic secretions.

As for to PI effects and mode of action, it would be of interest to investigate precisely the relation between the following elements: true ileal digestibility of dietary proteins, the quality of the amino acid supply, the proteases activities in the pancreatic secretions, the N apparent ileal digestibility.

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SUMMARY

In the European Community (EC), the plant and animal protein supply for animal production is covered for about 60% by imported protein sources as soya bean and soya bean meal, and for about 40% by protein sources grown in EC. The EC has therefore stimulated the growth of protein-rich seeds such as peas, lupins, beans and rapeseeds. A price-supporting policy engaged since 1978 resulted in a large increase in the production of peas in Europe from 1981 (363 000 tons) to 1992 (3 944 000 tons). However the incorporation of peas (*Pisum sativum*) in diets for young monogastric animals is limited in order to keep correct performance. Indeed in the present study, lower performance and lower digestibility with pea diets than with control diets were found in young piglets (Chapter III).
The aims of the research described in this thesis were to identify factors involved in pea digestion and to investigate on aspects of digestive processes affected by these factors.

Smooth seeded pea contains 40 to 50% starch, 20 to 25% crude protein, 10 to 15% non starch polysaccharides as the main chemical constituents. Crude protein consists of globulins (about 60% of CP), albumins (about 25% of CP) and insoluble proteins (about 15% of CP). The pea globulins are storage proteins of high molecular weights (> 150000 daltons). The pea albumins have low molecular weights (< 100000 daltons). They have biological activities due to their metabolic roles in the seeds. Activities specific to some of these albumins give them properties qualified as antinutritional. These proteins are therefore called antinutritional factors (ANFs). These include protease inhibitors (PI) which inhibit trypsin and chymotrypsin. This trypsin inhibitor activity (TIA) can be measured *in vitro*. The pea ANFs contain also lectins which form complexes with carbohydrates such as oligosaccharide moieties from the glycoproteins of the small intestinal mucosa. The PI can affect the N apparent digestibility by increasing the proportion of endogenous N via stimulation of pancreatic secretion. The size of the pancreas can also increase. But the mode of action of PI is submitted to many factors that may affect the effects on pancreas, as reviewed in chapter II. The PI have often been incriminated for the negative effects of peas, but this has never been shown directly. Lectins can also increase the endogenous N losses by increasing losses of mucus from the gastro-intestinal tract.

The methodology chosen to study the digestibility of pea and to study the effects of the major pea components on N apparent ileal digestibility can be described as follows. Pea protein (PP) isolates were produced from whole pea flour by precipitation. These isolates consisted mainly of globulin proteins and had no ANFs and carbohydrates. Albumin protein concentrates were

obtained by ultrafiltration to produce an ANF concentrate. Pea carbohydrate isolates were also produced from whole pea flour by precipitation.

The N apparent ileal digestibility of diets made with these fractions was determined in piglets (Chapter III). The N apparent ileal digestibility of the raw pea diets was 70% on average. The N apparent ileal digestibility of the pea protein isolate was 14 to 15 units higher than for the raw pea. The major proteins in peas (globulins) are therefore highly digestible. The addition of isolated pea carbohydrates to PP diets did not affect the N apparent ileal digestibility. The addition of 2.9% pea ANF concentrate to the pea protein isolate diet increased the TIA of the diet from 0.1 to 1.2 mg inhibited trypsin per g of feed, and the lectin content from 0.4 to 2.7 mg per g of feed. The N apparent ileal digestibility of the PP diet was then decreased by 7 units. When 0.6% of a different batch of pea ANF concentrate with a higher TIA than the previous one were added to a PP diet, no effect on the N apparent ileal digestibility was measured. However the TIA was increased from 0.1 to 0.8 mg inhibited trypsin per g of feed, and the lectin content from 1.5 to 1.9 mg per g of feed. The trypsin activity in the ileal chyme was also modified. From these results it was concluded that the biological active albumins in peas as found in the ANF concentrate, are partly responsible for the low N apparent ileal digestibility of raw peas. The albumin proteins would play a role through their protease inhibiting activity as well as through their structure that may resist to enzymic hydrolysis. However the share between both factors - protease inhibiting activity and structure - in the 7 units decrease could not be quantified.
It could also be concluded that diet composition is an important factor to consider when studying effects of ANFs, as addition of 0.6% of pea ANF concentrate did not affect the N apparent ileal digestibility of PP diet, although it did when added to a "raw pea + PP" diet.

Specific qualitative information on pea N digestibility were obtained through measurements of amino acid apparent ileal digestibility (Chapter IV). It was found that in raw pea, four essential amino acids (methionine, cystine, threonine, tryptophane) have a lower digestibility than the N digestibility. By removing the pea carbohydrates and the ANFs from the pea (PP diet), the digestibility of these amino acids was increased compared to the values for raw pea, especially for tryptophan (+20 to 30 units). The amino acid composition of the ileal digesta was used also to estimate the proportions of amino acid of "endogenous+bacterial" and dietary origins, by means of multiple regression analysis (Chapter IV). It was found that 70% to 80% of the AA in the ileal digesta of piglets fed the raw pea diet could be of "endogenous + bacterial" origin, and 20% to 30% of dietary origin and particularly of the albumin category. As for the PP diet, the amino acid in the ileal digesta would be almost exclusively of "endogenous + bacterial" origin. Based on these calculations, it was concluded that the true N

digestibility was approximately 90 - 95% for the raw pea, and close to 100% for the pea protein isolate.

As soya bean PI have been shown to affect pancreatic protease secretion in monogastric animals, the activities of pancreatic proteases (trypsin and chymotrypsin) were measured in different parts of the gastro-intestinal tract, upon feeding raw pea or PP diets, with or without the addition of pea ANF concentrate. The activities were measured in the ileo-caecal digesta (Chapter V), in the jejunal digesta (Chapter V), in the pancreatic secretions (Chapter VI) and in the pancreatic tissue (Chapter VI). The total protease activities in the pancreatic juice collected totally over 12 hours were equivalent for the raw pea and the PP diets although the TIA was higher in the former diet (Chapter VI). In the pancreas taken 3.5 hours after feeding, the activities were lower for the raw pea than for the PP diet (Chapter VI). The addition of pea ANF concentrate to a PP diet or to a " raw pea + PP" diet did not affect protease activities in the pancreas taken 3.5 hours after feeding. The enzyme activities in the pancreas from animals in an unfasted state are the result of simultaneous secretion and biosynthesis. The low apparent ileal digestibility of some essential amino acids of the raw pea diet was suspected to have limited the biosynthesis of enzymes and therefore, to be involved in the lower activity found in the pancreas for the raw pea diet, compared to the PP diet. The addition of 0.6% pea ANF concentrate to the PP diet decreased the activities at the jejunal level but increased the activities at the ileo-caecal level (Chapter V). The activities at the ileo-caecal level were higher with the "raw pea + PP" diet than with the PP diet. The differences could have been due to differences in the rates of breakdown of enzyme activity. Other hypotheses are also discussed (Chapter V). The addition of 0.6% ANF concentrate to the "raw pea + PP" diet did not change protease activities at the end of the ileum.

From this set of results, it was concluded that pancreatic protease activities measured at different sites of the gastro-intestinal tract cannot be related to the N apparent ileal digestibility. In addition, the ranking of the activities for different diets at one site is not the same at another site of the gastro-intestinal tract.

A general concept of PI effects and PI mode of action was proposed (Chapter VII). When the level of active proteases in the upper small intestine after partial inhibition is still high enough to hydrolyse the dietary proteins, the pancreas would not be stimulated to produce more enzymes. However, when the amount of active proteases, after partial inhibition by PI, is lower than the demand for hydrolysing the dietary proteins, the pancreas could be stimulated to secrete more enzymes. However, if the supply of dietary amino acid does not meet the quantitative and qualitative requirements for extra-synthesis of enzymes, the pancreas would not be able to respond to the stimulation and to maintain a sufficient biosynthesis of proteases.

As for elucidation of pea digestibility, half of the difference in N apparent ileal digestibility between raw pea and pea protein isolate has still to be explained. The fact that "reconstituted"

pea did not give the low N apparent ileal digestibility of the raw pea is probably mainly due to the fact that PP was not the whole pea proteins and that the structure of the cotyledon cells was disrupted. These elements and others are discussed in chapter VII.

SAMENVATTING

In de voorziening van plantaardig eiwit voor dierlijke produktie in de EEG wordt ongeveer 58% van de benodigde hoeveelheid (voornamelijk als sojabonen en sojaschroot) geïmporteerd van buiten de EEG. In 40% wordt voorzien door eigen EG produktie. Vanaf 1978 is de EEG begonnen met ondersteuning van de teelt van gewassen die als vervanger voor de import van soyaprodukten kunnen dienen. Dit heeft geleid tot een sterke toename in de teelt van erwten. In 1981 was de produktie 363.000 ton en in 1992 3.944.000 ton. De vervanging van soja schroot door erwten (*Pisum sativum*) heeft enkele negatieve effekten in de voeding en produktie van met name éénmagige landbouwhuisdieren. Bij opname van erwten in het rantsoen wordt soms lage schijnbare vertering van eiwit en lagere groei gevonden.
In de literatuur en voorstudies werd gevonden dat de schijnbare ileale verteringscoëfficiënten van N en de groei van biggeb lager was dan op het controlerantsoen zonder erwten (Hoofdstuk III). In het onderzoeksprogramma was het doel die faktoren te indentificeren die samenhangen met het verteringsproces en die verantwoordelijk zijn voor deze lagere ileale N vertering.

Erwten bevatten 40 - 50% zetmeel, 20 - 25% ruw eiwit en 10 - 15% nietzetmeel polysachariden. De ruw eiwit fractie bestaat uit globulinen (ongeveer 60%), albumine (ongeveer 25%) en nog 15% onoplosbaar eiwit. Globuline eiwitten worden als reserve eiwit gekarakteriseerd en hebben een hoog molecuul gewicht (> 150.000 dalton). Albumine uit erwten hebben een lagere molecuul gewicht (< 100.000 dalton). Deze laatste omvatten ook zogenaamde antinutritionele faktoren (ANF's). O.a. protease inhibitoren (PI). Deze remmen de werking van de pancreas enzymen Trypsine (T) and Chymotrypsine (CT). De totale remmingsactiviteit (TIA) wordt *in vitro* bepaald als som van geremde Trypsine and Chymotrypsine. Erwten ANF's omvatten ook lectines die zich binden aan het koolhydraten deel in de mucus van de darmwand.
De protease inhibitoren (PI) in erwten kunnen de schijnbare ileal vertering van rantsoen N beïnvloeden (verminderen) door remming van de werking van Trypsine en Chymotrypsine. Als gevolg van de remming wordt de pancreas gestimuleerd door meer secretie van proteases in het darmlumen en hiermee meer endogene eiwitten.
De literatuur over de verschillende effekten van PI is in hoofdstuk II gereviewed. In de literatuur wordt algemeen PI verantwoordelijk gesteld voor de lage erwten N verteerbaarheid. Dit is voor erwten echter nooit specifiek aangetoond.

De methoden die gevolgd zijn om de lage schijnbare ileale N vertering te bestuderen en te verklaren bestonden uit het scheiden van erwten in fractiesen het bepalen van de ileale N verteerbaarheid bij elk van deze fracties in het rantsoen.
De bestudeerde erwten fracties waren:

- geïsoleerde erwten eiwit (PP) verkregen door precipitatie van erwten eiwit uit erwtenmeel. Deze fractie bestaat in hoofdzaak uit globulinen zonder de ANF fractie,
- geïsoleerde koolhydraten ook verkregen door precipitatie uit erwtenmeel,
- ANF's zijn geconcentreerd aanwezig in de door ultrafiltratie verkregen albumine fractie.

In hoofdstuk III zijn de proeven beschreven waarin de schijnbare ileale N vertering gemeten werd bij biggen die rantsoenen met deze fracties kregen. De resultaten laten zien dat de schijnbare ileale N vertering van rauwe erwten ongeveer 70% was. De schijnbare ileale N verteerbaarheid van PP was 14 tot 15 eenheden (%) hoger dan bij rauwe erwten (Hoofdstuk III). Dit betekent dat de geïsoleerde erwten globuline fractie zeer goed verteerbaar is. Toevoeging van 2.9% ANF bevattend concentraat aan het PP rantsoen verlaagde de ileal N vertering met 7 eenheden in vergelijking met PP eiwitten alleen. Wanneer echter ANF's (0.6%) uit een andere erwtenbatch werd gebruikt (met meer trypsine inhibitor activiteit (TIA)) waardoor de TIA activiteit in het proefvoer vergelijkbaar was met de toevoeging van 2.9% ANF concentraat, aan het PP rantsoen, veranderde de ileale N vertering in N echter nauwelijks. Bij toevoeging van dit ANF concentraat aan een rantsoen met rauwe erwten + PP werd wel een negatief effect gevonden. Dit betekent dat de biologische active albumine fractie uit erwten minstens ten dele verantwoordelijk is voor de lage ileale N verteerbaarheid van volledige erwten. De structuue en de TIA activiteit uit deze ANF's fracties zijn dus minstens voor 7 eenheden verantwoordelijke voor de lagere N verteerbaarheid van rauwe erwten. De overblijvende 7% hebben te maken met andere factoren. De aparte bijdrage van structuur en activiteit kan door de gekozen proefopstelling niet worden bepaald.
Wel betekenen de resultaten dat de totale rantsoensamenstelling (+structuur+activiteit van o.a. eiwitten) belangrijk zijn.
De specifieke bijdrage van ANF's aan de ileale eiwitverstering werd daarop nagegaan door meeting van schijnbare ileale aminozuren verteerbaarheid. (Hoofdstuk IV). Speciaal de aminozuren verteerbaarheid van methionine, cystine, threonine en tryptophan was lager dan totaal N bij de rauwe erwten. Met het PP was de verteerbaarheid van deze vier aminozuren veel hoger dan in de rauwe (met 20 - 30 eenheden). Daarop werd met behulp van een multiple regressie van op de aminozuur samenstelling van endogeen eiwit (a), bacterieel eiwit (b) en voereiwit (c) nagegaan welke de bijdrage van a,b,c aan ileaal eiwit kon zijn (Hoofdstuk IV). Resultaten toonden aan dat bij voedering van rauwe erwten 70 tot 80% van het van het ileale

chymus eiwit afkomstig kan zijn bij van endogeen + bacterieel eiwit, het erwteneiwit (voornamalijk het albuminedeel) kan zo 20 - 30% bijdragen aan chymuseiwit.
Chymus van varkens gevoerd met PP rantsoen kwam qua aminozuursamenstelling bijna volledig overeen uit een combinatie van endogeen en bacterieel eiwit.

In de literatuur (Hoofdstuk II) kwam naar voren dat soya de PI de secretie van pancreas eiwit (Trypsine + Chymotrypsine) bij biggen kan beïnvloeden. Daarom werd dit aspect ook bij erwten nagegaan. Biggen werden gevoerd met erwten en/of PP en ook met erwten + ANF en PP + ANF. In pancreasweefsel, pancreassecreet, jejunum chymus en ileale chymus werden de activiteiten van T en CT gemeten (Hoofdstuk V en VI).
De activitieten van T en CT in het pancreas secreet waren gelijk in dieren op rauwe erwten en op PP rantsoen ondanks de hogere TIA activiteit bij rauwe erwten (Hoofdstuk VI). In het pancreasweefsel waren de activiteiten T en CT, gemeten 3.5 uur na voeren, lager bij dieren op rauwe erwten dan bij dieren op PP diet (Hoofdstuk VI).
Toevoeging van ANF's aan erwtenrantsoen (rauwe erwten + ANF) of aan rantsoen met geïsoleerd erwten eiwit (PP+ANF) beïvloeden de T en CT activiteiten in het pancreasweefsel verder niet. Toevoeging van ANF's aan PP rantsoen verlaagde de T en CT activiteit in jejunum chymus maar gaven hogere activiteiten in ileum chymus (Hoofdstuk V). In ileale chymus van dieren gevoerd met rauwe erwten + PP was de T en CT activiteit hoger dan op PP rantsoen. Toevoegingen ANF aan dit rantsoen veranderde de activiteit van de enzymen in de chymus echter niet.
Uit deze proeven werd geconcludeerd dat de activiteit van T en CT in chymus op verschillende plaatsen in het maagdarmkanaal niet aan de schijnbare ileale N vertering kan worden gerelateerd. Bovendien was de activiteit van de enzymen in de chymus van verschillende darmdelen niet met elkaar gecorreleerd. Op grond van de bevindingen van Hoofdstuk III t/m VI werd in Hoofdstuk VII een concept ontwikkeld om de werking van erwten PI te verklaren. Dit concept houdt in dat wanneer de activiteit van proteases in chymus maar gedeeltelijk geremd is en er toch voldoende is voor hydrolyse van voedingseiwitten er geen stimulatie tot secretie van pancreas proteases hoeft op te treden. Er worden dan ook geen extra T en CT geproduceerd. Wanneer echter de inhibitor door PI zodanig is dat er te weinig proteases zijn voor hydrolyse van voereiwit wordt de pancreas wel gestimuleerd tot extra secretie van T en CT. Wanneer uit rantsoen echter te weinig aminozuur voor de synthese van pancreas enzymen worden geabsorbeerd kan de pancreas niet aan de extra behoefte voldoen. Als gevolg daalt de biosynthese van proteases.
Wat betreft de effecten op schijnbare ileale N vertering blijft nog een gedeelte van de lagere vertering onverklaard. Geïsoleerde erwten koolhydraten zijn voor deze daling in ileale N vertering niet verantwoordelijk. De samenvoeging van PP, koolhydraten van ANF geven een

betere N vertering dan rauwe erwten. Mogelijk heeft de structuur van erwteneiwit die door de isolatie van de fracties wordt veranderd hiermee te maken. Een discussie punt hierbij is in hoeverre een mogelijke antigeniteit van het erwteneiwit hierin een rol speelt. Dit is in Hoofdstuk VII bediscurrieerd.

RESUME

La production de protéines végétales et animales pour l'alimentation animale en Europe est assurée pour 60% par l'importation de sources protéiques tel que le soja, et pour 40% par la production européenne de protéines. La communauté européenne a donc incité à la production de plantes riches en protéines tel que le pois, le lupin, le haricot. La politique de support des prix engagée depuis 1978 s'est traduite par une augmentation importante de la production de pois protéagineux en Europe de 1981 (363 000 tonnes) à 1992 (3 944 000 tonnes). L'incorporation de pois protéagineux (*Pisum sativum*) dans des aliments pour monogastriques est cependant limitée pour éviter des problèmes zootechniques. En effet dans cette étude, les performances de croissance et les digestibilités ont été plus faibles chez les porcelets consommant du pois cru que chez ceux recevant un régime témoin (Chapitre III).
Les objectifs des travaux de recherche rapportés dans cette thèse étaient l'identification des facteurs impliqués dans la digestion du pois et l'étude de divers aspects concernant des processus digestifs affectés par ces facteurs.

Le pois protéagineux à graine lisse contient 40 à 50% d'amidon, 20 à 25% de matière azotée totale, 10 à 15% de polysaccharides non amylacés, pour les constituants chimiques principaux. Les protéines de pois sont de 3 types: les globulines (environ 60% de la MAT), les albumines (environ 25% de la MAT) et les protéines insolubles (environ 15% de la MAT). Les globulines de pois sont des protéines de réserve à poids moléculaire élevé (> 150000 daltons). Les albumines de pois sont des protéines de faible poids moléculaire (< 100000 daltons) à activités biologiques. Des activités spécifiques à certaines albumines leur confèrent des propriétés qualifiées d'antinutritionnelles. Ces protéines sont alors appelées facteurs antinutritionnels (FAN). Les FAN de pois comprennent des inhibiteurs de protéases qui inhibent la trypsine et la chymotrypsine. L'activité antitrypsique peut être mesurée *in vitro*. Les FAN de pois contiennent aussi des lectines qui ont la propriété de se complexer aux oligosaccharides, tels les oligosaccharides des glycoprotéines de la muqueuse intestinale. Les facteurs antitrypsiques peuvent affecter la digestibilité apparente de l'azote en augmentant la proportion d'azote endogène par une stimulation des sécrétions pancréatiques. La taille du pancréas peut aussi augmenter. Mais le mode d'action des facteurs antitrypsiques est soumis à plusieurs facteurs de variation qui peuvent influencer les effets sur le pancréas (Chapitre II). Les facteurs antitrypsiques ont souvent été soupçonnés d'être responsables des effets négatifs du pois chez les monogastriques. Ceci n'a cependant jamais été démontré directement. Les lectines peuvent aussi augmenter les pertes d'azote endogène sous forme de mucus du tube digestif.

La méthodologie choisie pour étudier la digestibilité du pois et les effets de ses constituants majeurs sur la digestibilité iléale apparente de l'azote peut être schématisé de la façon suivante. Des isolats de protéines de pois (PP) ont été produits par précipitation à partir de farine de pois cru. Ces isolats contenaient principalement des globulines, sans FAN et glucides de pois. Des concentrés d'albumines ont été produits par ultrafiltration pour fournir des concentrés de FAN. Des isolats de glucides de pois ont aussi été produits par précipitation à partir de la farine de pois cru.

La digestibilité apparente de l'azote des régimes constitués avec ces différentes fractions a été déterminée chez des porcelets (Chapitre III). La digestibilité iléale apparente de l'azote des régimes à base de pois cru était de 70%. La digestibilité iléale apparente de l'azote des régimes à base d'isolat protéique était supérieure de 14 à 15 points. Les protéines majeures de pois, c'est à dire les globulines, sont donc très digestibles. L'addition de glucides isolés de pois n'a pas affecté la digestibilité iléale apparente de l'azote des isolats protéiques. L'addition de 2.9% de concentré de FAN au régime à base d'isolat protéique a augmenté l'activité antitrypsique du régime de 0.1 à 1.2 mg de trypsine inhibée par g d'aliment, et la teneur en lectines de 0.4 à 2.7 mg par g d'aliment. La digestibilité iléale apparente de l'azote du régime PP a alors baissé de 7 points. Quand 0.6% d'un autre concentré de FAN de pois ayant une plus forte activité que le concentré précédent ont été ajoutés à un régime PP, la digestibilité de l'azote n'a pas été affectée. Cependant, l'activité antitrypsique de l'aliment avait augmentée de 0.1 à 0.8 mg de trypsine inhibée par g d'aliment, et la teneur en lectines de 1.5 à 1.9 mg par g d'aliment. L'activité de la trypsine pancréatique dans les digesta iléaux a aussi été modifiée. Il a été conclu de ces résultats que les albumines de pois constituant les protéines des concentrés de FAN sont partiellement responsables de la faible digestibilité iléale de l'azote du pois cru. Les albumines agiraient à la fois par leur activité antitrypsique et par leur structure moléculaire leur conférant une résistance à l'hydrolyse enzymatique. La part de responsabilité entre ces deux éléments, c'est à dire l'activité antitrypsique et la structure moléculaire, dans la baisse de 7 points de digestibilité n'a cependant pu être quantifiée. Il a par ailleurs été constaté que la composition alimentaire est un facteur important à considérer dans les études nutritionnelles des FAN. En effet l'addition de 0.6% de concentré de FAN de pois n'a pas affecté la digestibilité iléale apparente du régime PP, mais a réduit celle d'un régime à base de pois cru et d'isolat protéique.

La digestibilité iléale apparente des acides aminés des régimes de pois cru ou d'isolat protéique a été déterminée (Chapitre IV). Quatre acides aminés (méthionine, cystine, thréonine, tryptophane) du régime à base de pois cru avaient une digestibilité plus faible que celle de l'azote du régime. En éliminant les glucides de pois et les FAN (régime PP), la digestibilité de ces acides aminés a augmentée, surtout celle du tryptophane (+20 à 30 points).

Les proportions d'acides aminés d'origine "endogène + bactérienne" et d'origine alimentaire ont été calculées par régression multiple, à partir de la composition en acides aminés des digesta iléaux et de protéines de références (Chapitre IV). Les résultats de cette analyse indiquent que 70% à 80% des acides aminés des digesta iléaux de porcelets consommant les régimes de pois cru seraient d'origine "endogène + bactérienne", et 20% à 30% d'origine alimentaire et plus particulièrement de la catégorie des albumines. Les acides aminés des digesta iléaux des porcelets consommant les régimes PP seraient exclusivement d'origine "endogène + bactérienne". D'après ces calculs, la digestibilité vraie de l'azote du pois cru serait de 90% à 95%, et celle de l'azote de l'isolat protéique serait aux environs de 100%.

Comme les facteurs antitrypsiques du soja affectent la sécrétion de protéases pancréatiques chez les monogastriques, l'activité de la trypsine et de la chymotrypsine a été mesurée à différents niveaux du tube digestif pour étudier les effets des régimes à base de pois cru ou d'isolat protéique, avec ou sans concentré de FAN de pois. Les activités ont été mesurées dans les digesta iléaux (Chapitre V), dans les digesta jéjunaux (Chapitre V), dans les sécrétions pancréatiques (Chapitre VI), et dans le tissu pancréatique (Chapitre VI). L'activité totale des protéases dans les sécrétions pancréatiques collectées totalement pendant 12 heures était équivalente pour le régime de pois cru et pour le régime PP (Chapitre VI). L'activité antitrypsique était cependant plus élevée dans le régime à base de pois cru. Dans le pancréas collecté 3.5 heures après l'alimentation, les activités étaient plus faibles pour le régime à base de pois cru que pour le régime PP (Chapitre VI). L'addition de concentré de FAN de pois au régime PP ou au régime "pois cru + PP" n'a pas affecté l'activité des protéases dans le pancréas collecté 3.5 heures après le repas. L'activité enzymatique mesurée dans le pancréas d'animaux non à jeun est le résultat de la sécrétion et de la biosynthèse simultanée. La faible digestibilité iléale apparente de certains acides aminés essentiels du régime à base de pois cru a pu limiter la biosynthèse simultanée d'enzymes, et donc être en partie responsable de la plus faible activité dans le pancréas pour ce régime. L'addition de 0.6% de concentré de FAN au régime PP a fait baisser les activités de la trypsine et de la chymotrypsine dans le contenu du jéjunum, et a fait augmenter celles du contenu en fin d'iléon (Chapitre V). Les activités dans le contenu en fin d'iléon étaient plus élevées avec le régime "pois cru + isolat protéique" qu'avec le régime PP. Les différences peuvent être liées à des différences de vitesse de dégradation des activités enzymatiques. D'autres hypothèses sont aussi discutées (Chapitre V). L'addition de 0.6% de concentré de FAN au régime à base de "pois cru + isolat protéique" n'a pas changé l'activité des protéases dans le contenu en fin d'iléon.

A partir de cet ensemble de résultats, il a été conclu que les activités des protéases pancréatiques mesurées à différent niveaux du tube digestif ne sont pas corrélées à la digestibilité iléale apparente de l'azote. De plus, la hiérarchie des activités correspondant à différents régimes, à un niveau du tube digestif n'est plus la même à un autre niveau.

Un modèle général sur les effets des facteurs antitrypsiques et leur mode d'action est proposé (Chapitre VII). Quant le niveau de protéases actives dans le tube digestif proximal, après inhibition partielle par les facteurs antitrypsiques, est encore suffisant pour hydrolyser les protéines alimentaires, le pancréas ne serait pas stimulé pour une sécrétion accrue de protéases. Par contre, quand le niveau de protéases actives, après inhibition partielle par les facteurs antitrypsiques, ne satisfait plus la demande pour une hydrolyse maximum des protéines alimentaires, le pancréas serait stimulé pour sécréter plus d'enzymes. Si cependant l'apport en acides aminés alimentaires ne satisfait pas les besoins quantitatifs et qualitatifs pour une synthèse accrue d'enzymes, le pancréas ne serait pas en mesure de répondre à la stimulation et de maintenir une biosynthèse de protéases suffisante.

Quant à l'explication de la digestibilité du pois, la moitié de la différence de digestibilité iléale apparente de l'azote entre le pois cru et l'isolat protéique doit encore être déterminée. Divers facteurs telle que la structure intrinsèque des cellules des cotylédons de pois qui est éliminée en reconstituant le pois à partir de ses fractions majeures sont discutés au chapitre VII.

List of publications

Doreau, M.; Le Guen, M.P.; Poncet, C. (1987) Vitesse de renouvellement du liquide du rumen mesurée par le polyethylène glycol et le Co-EDTA à trois niveaux du tube digestif. *Reproduction Nutrition Development* 27, 227-228

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Curriculum vitae

Marie-Pierre Le Guen was born September 27, 1963 in Lannion, France. She received in 1985 the diploma from Ecole Nationale Supérieure Féminine d'Agronomie in Rennes (France), with a specialisation in Animal Production. In october 1985, she entered the University of Grenoble (France) for a diploma in Computer Science she received in october 1986.

From **january 1987** until **october 1988**, she has been working on protein structure in the Department of Molecular Biology and on data compression in the Department of Computer Science, at the University of Brandeis, in Waltham, Massachussets, USA.

From **january 1989** until **may 1993**, she has been working for EURETEC in France, on the EUREKA-IMPROFEED project aiming at improving the nutritional value of legume seeds and oilseeds for monogastric animals. During this period, she carried out research experiments on peas in Wageningen. This resulted in the present thesis.

From **june 1993**, she is working for EURETECII (France) on the research program EUREKA-EUROPROTEINS aiming at improving the nutritional value of legume seeds and oilseeds for ruminants and poultry.

STELLINGEN

I.

Reconstitution of pea seed by association of its isolated major components do not represent the whole seed on a nutritional point of view. (this thesis)

II.

The apparent nitrogen ileal digestibility of raw peas can be low in piglets. (this thesis)

III.

The pancreatic protease activities in the ileal chyme of piglets are not correlated with activities at other sites of the gastrointestinal tract. They are not correlated either with the apparent nitrogen ileal digestibility. (this thesis)

IV.

The quality of the dietary proteins, in terms of amino acid digestibility for instance, is an important factor affecting pancreatic protease activity. (this thesis)

V.

The reasons for the unsatisfactory digestive utilization of peas in piglets has not been completely clarified. (this thesis)

VI.

Research ends up in more research.

VII.

The ideal ambassador: courteous as a French, generous as a Dutch (Computer Science newspaper: Genesis Today, 1992)

VIII.

About three hundred possibly dangerous chemicals can get into the Dutch water supply; tests can be done to check for only ninety (The New Yorker, august 12, 1991). Would French wine be safer?

IX.

"Manure is not a holy thing but were it lies there come miracles". "Mest is geen heiligheijt maar doet mirakelen waar hij leit" (old Dutch saying).

X.

Rauwe erwten zijn niet altijd goed voor biggen, maar mischien is erwtensoep dat wel.

XI.

A child-to-be is a sure way to respect deadlines, and a good stimulant to accomplish even a tedious work.

Marie-Pierre Le Guen,
Pea proteins for piglets: effects on digestive processes
Wageningen, The Netherlands, 6 december 1993