POTENTIAL BIOTECHNOLOGICAL APPLICATIONS OF AN ARTIFICIAL RUMEN SYSTEM

Els Geertman

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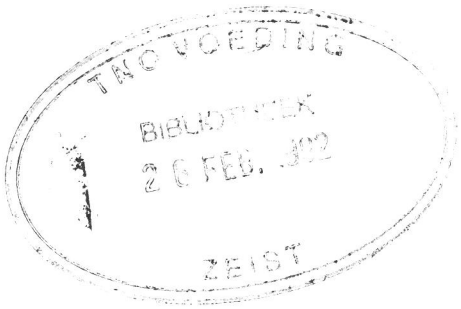

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CONTENTS

ABBREVIATIONS

A	acetic acid
ADF	acid detergent fibre
B	butyric acid
CMC	carboxymethylcellulose
CS	cell solubles
FPC	filter paper cellulose
IB	isobutyric acid
IV	isovaleric acid
LPRM	low protein rapeseed meal
LPSM	low protein soybean meal
LR	loading rate
MV	methylvaleric acid
NDF	neutral detergent fibre
P	propionic acid
PNPG	p-nitrophenyl-β-D-glycopyranoside
RM	rapeseed meal
SM	soybean meal
TS	total solids
V	valeric acid
VFA	volatile fatty acids
VOT	L-5-vinyloxazolidine-2-thione
VS	volatile solids

1

GENERAL INTRODUCTION

In herbivorous animals a symbiotic relationship exists between host and microbes housed in the gut. The microorganisms convert plant structural polysaccharides, which cannot be degraded by animal digestive processes, to products which the host animal can utilize for energy and growth (Hobson & Wallace, 1982a).

The particular conditions developed in the herbivores using microbes to ferment plant fibres vary from one species to another (Moir, 1968), leading to selection of microbial populations unique for each animal type. Bacteria occur among all gut mutualistic populations, protozoa are prominent in many, and even anaerobic fungi may be important (Orpin, 1977a). In some animals the microbial digestion occurs in a compartment preceding the stomach in which pepsin and acid are secreted (pre-peptic), in others the microbial attack follows the action of the host enzymes (post-peptic). In both instances the acidity of the animal's peptic secretions is sufficient to kill most of the microbes subjected to it. Some of the animals in which a mutualistic relationship with a gut microbial population has developed are listed in Table 1 (Hungate, 1988).

The pre-peptic location makes the host completely dependent on its symbionts because they ferment all ingested foods, whereas with the post-peptic location any products from the animal's digestive enzymes can be absorbed prior to the microbial action. The rumen is a large portion of the stomach in which the ingesta are held for pre-peptic microbial action; an enlarged caecum, or a capacious large-intestine, retains the digesta during post-peptic fermentations (Hungate, 1988).

In the rumen microorganisms degrade plant polymers to end products of anaerobic fermentation and microbial biomass. The host animal can use these end products, mainly volatile fatty acids, as carbon and energy sources. The microbial cells serve as a source of protein, vitamins and other nutrients (Hungate, 1966; Wolin, 1981; Lin et al, 1985).

The ruminant stomach consists of four compartments : the rumen, the reticulum, the omasum and the abomasum (Fig. 1).

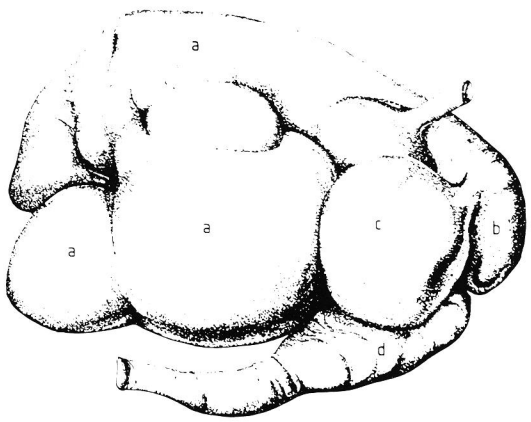


Fig. 1. The four compartments of the ruminant stomach (a : rumen, b : reticulum, c : omasum, d : abomasum).

Table 1. Animals with nutritionally mutualistic microbes (including fungi) (Hungate, 1988)

Animals	Microbes	Location
Wood roach, *Cryptocercus punctulatus*	Flagellate protozoa	Post-peptic
Termites	Flagellates in some, bacteria in some, fungi in some	Post-peptic
Birds, grouse and ptarmigan	Bacteria	Post-peptic
Marsupial mammals (quokka, kangaroo and wallaby)	Ciliate protozoa and bacteria	Pre-peptic
Leaf-eating colobid and langur monkeys	Bacteria	Pre-peptic
Sloths	Bacteria	Pre-peptic
Rodents	Bacteria	Post-peptic with coprophagy
Rabbit and hare	Bacteria	Post-peptic with coprophagy
Elephant and hyrax	Bacteria and ciliates	Post-peptic
Hippopotamus and peccary	Bacteria and ciliates	Pre-peptic
Dugong and manatee	Bacteria	Post-peptic
Camel, llama and alpaca	Bacteria and ciliates	Pre-peptic
Horse, zebra, tapir and rhinoceros	Bacteria and ciliates	Post-peptic
Cattle, sheep and other ruminants	Bacteria, ciliates and microscopic fungi	Pre-peptic

The fermentation occurs in the reticulum and the rumen. In the omasum the liquid content of the digesta passing into the abomasum is reduced by the removal of water and fermentation products produced in the rumen are absorbed (Baldwin, 1984). Peptic digestion of protein in the abomasum, the acid stomach, is followed by tryptic digestion when the digesta enter the duodenum (Hungate, 1988).

Food enters the reticulo-rumen together with large volumes of saliva containing bicarbonate and phosphates (Fig. 2). The rumen is large in relation to the body size : approximately 5 to 10 l in sheep and 100 to 150 l in cattle. At the passage from the rumen a selection of particle sizes occurs. Liquid remains in the rumen for 10-24 hours, large food particles are held in the rumen for 2-3 days. The flow of digesta through the rumen is mediated by the saliva produced and by the water intake of the animal. The relatively high concentration of sodium bicarbonate in the saliva prevents acidification of the rumen content by the fermentation products. The gas phase consists of carbon dioxide (65 %) and methane (35 %), although trace amounts of other gases (H_2, N_2, O_2) have been reported (Wolin, 1979). The small amounts of oxygen that enter the rumen are rapidly reduced by a small fraction of the gut bacteria. The contents of the

rumen are mixed regularly by contraction and relaxation of the rumen wall (Hobson & Wallace, 1982a).

Some of these rumen features are constant to almost all ruminants and feeding situations. The environment is anaerobic with a very low redox potential (-350 mV) and a temperature of 39-41°C. The pH is maintained fairly constant (pH 6-7) by the buffering capacity of bicarbonate and the absorption of fermentation products through the rumen wall. Most microorganisms in the rumen will thus be obligate anaerobes with an optimum pH for growth of 6.5 and an optimum growth temperature of 39°C (Counotte, 1981). The retention time of solids is approximately three times longer than the retention time of liquids (Hungate, 1966; Wolin, 1979; 1981).

Microorganisms in the rumen

Over 200 species of bacteria have been isolated from the rumen and 20 to 30 of these can be found in high numbers (> 10^7 per ml). The total number of viable bacteria in the rumen can amount to 10^{10} to 10^{11} cells per ml (Hungate, 1966, Wolin, 1981). The activity of the rumen bacterial flora is not constant, but varies according to the changing conditions in the gut. Furthermore the rumen is compartmentalised, different bacterial populations being associated with feed particles, the rumen wall and the liquid phase of the rumen contents.

A wide range of aerobic, facultative aerobic and obligate anaerobic bacteria is found in the rumen (Stewart & Bryant, 1988). Cellulolytic bacteria, hemicellulolytic bacteria, starch-digesting bacteria, bacteria fermenting mono- and disaccharides, proteolytic bacteria, lipolytic bacteria and the bacteria utilizing end-products of other bacteria (acid-utilizing and methanogenic bacteria) form the main groups of bacteria present in the rumen (Counotte, 1981).

In addition, about 20 species of protozoa are present in the rumen. Although occurring at much lower numbers (10^5 to 10^6 per ml) than bacteria, these large microorganisms can account for up to half of the ruminal biomass under certain dietary conditions (Williams, 1986). The majority of the protozoa are ciliates, although flagellates were also found in the rumen (Clarke, 1977; Williams & Coleman, 1988; Prins, 1990). The ciliates can be divided in two groups : entodiniomorphs and holotrichs. Although the entodiniomorph ciliates are able to take up soluble compounds, they feed principally by the engulfment of particulate matter (Williams & Coleman, 1988). The holotrich ciliates are mainly involved in the metabolism of soluble sugars and occur in the highest numbers when soluble carbohydrates are readily available in the diet (Hungate, 1966).

Phycomycetous fungi constitute the third group of microorganisms in the rumen. The life cycle of these organisms consists of an alternation of generations between a motile zoospore stage free in the rumen liquor, and a non-motile vegetative reproductive stage which occurs on the digesta particles (Orpin, 1975; 1976; 1977b). Many species are known and all were shown to ferment plant cell wall polysaccharides as sole sources of carbon and energy (Orpin, 1984). These anaerobic rumen fungi are estimated to represent about 8 % of the total rumen biomass (Citron et al, 1987).

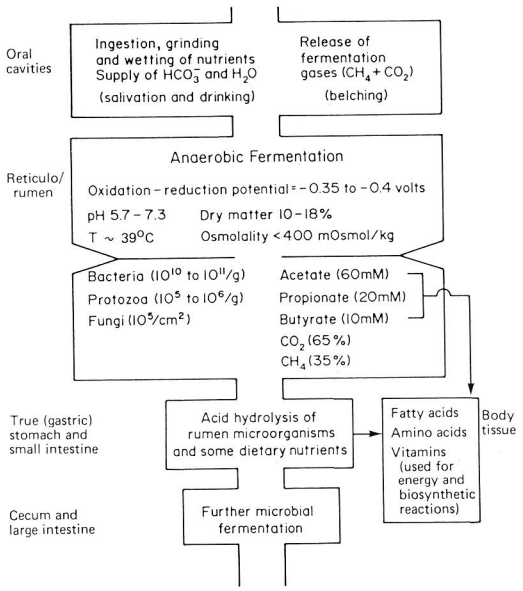


Fig. 2. Schematic representation of the ruminant as a factory for the conversion of food to animal products (Wolin, 1981; Lin et al, 1985). Reproduced with permission of Butterworth - Heinemann Publishers.

Most microorganisms in the rumen are attached to and move with the solids. In this way they can survive in the rumen despite their relatively low growth rates (Prins & Clarke, 1980).

Metabolism in the rumen

Polymers, the major organic components of plants, undergo extensive degradation in the rumen. About 70 to 85 % of the total dietary organic matter is degraded. Volatile fatty acids, principally acetic acid (56-70 %), propionic acid (17-29 %) and butyric acid (9-19 %), together with methane and carbon dioxide are the main fermentation products in the rumen (Hungate, 1966; Wolin, 1981). Other fermentation products such as succinate and hydrogen are also produced in large quantities, but they do not accumulate because they are converted rapidly to end products such as propionic acid and methane, respectively (Wolin & Miller, 1983). Minor intermediates, as lactic acid, formate and ethanol, are also rapidly converted to volatile fatty acids,

carbon dioxide and methane (Hungate, 1966). The volatile fatty acids are transported from the rumen into the blood stream and used as the major source of carbon and energy for metabolic activities of the animal (Wolin, 1979; Counotte, 1981). Both methane and carbon dioxide are removed from the rumen by belching (Wolin, 1981; Lin et al, 1985). The production of methane is usually considered a loss of energy for the ruminant (Wolin, 1979).

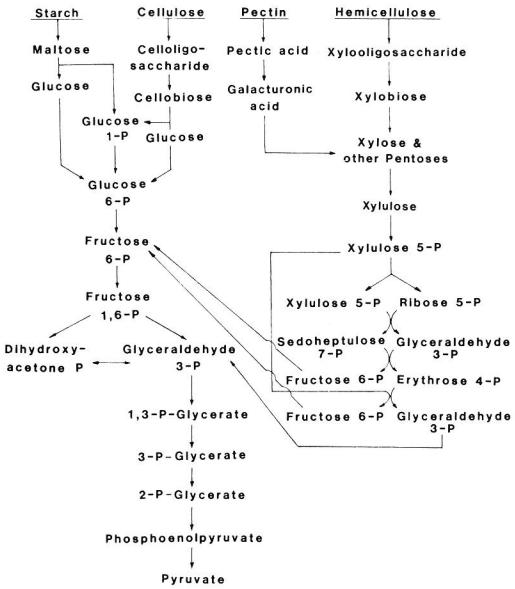


Fig. 3. Metabolic pathways of the major carbohydrate constituents of plants to pyruvate in the rumen (Lin et al, 1985). Reproduced with permission of Butterworth - Heinemann Publishers.

Plant carbohydrates are the predominant components of diets fed to ruminants. The majority of these carbohydrates enter the rumen in the form of insoluble polymers. Degradation of these polymers takes place in three sequential stages : dissociation from the plant structural matrix, followed by hydrolysis into small saccharides and finally fermentation of these saccharides.

Most polysaccharides entering the rumen can be considered as belonging to one of two general types : plant storage polysaccharides, such as starch, or plant structural polysaccharides, such as cellulose, hemicellulose, pectin and lignin. Storage polysaccharides are easily degraded by rumen microorganisms, whereas structural polysaccharides, fibres, are more resistant to microbial attack. Lignin, an inert polymer

based on phenyl-propane units present in secondary thickened cell walls, is bound extensively to the matrix-polysaccharides of the cell wall forming a lignin-carbohydrate complex. Lignin degradation is essentially an aerobic process and is unlikely to occur to any significant extent in the rumen (Chesson & Forsberg, 1988). The conversion of radiolabelled lignin substrates to gaseous products under anaerobic conditions is very slow, only 2 to 4 % in about ten months (Benner et al, 1984; Benner & Hodson, 1985). Lignin can be solubilised to a great extent in the rumen, but it appears that the solubilised lignin is not further degraded to gaseous end products (Gijzen et al, 1986; Akin & Benner, 1988).

Starch, a homopolymer consisting of α-1-4 linked glucose residues, is readily utilised by rumen microorganisms (Chesson & Forsberg, 1988). Many strains of ruminal bacteria, protozoa and fungi produce amylases, enzymes needed to degrade starch (Coleman, 1986; Williams, 1986; Citron et al, 1987; Stewart & Bryant, 1988, McAllister et al, 1990).

Cellulose, a linear homopolymer of β-1-4 linked glucose, containing up to 14,000 units, forms tightly packed parallel molecules, linked together by hydrogen bonds. The resulting micro-fibrils with variable degree of ordering (crystallinity) are embedded in a giant ligno-(hemi)cellulose macro-molecule in plant cell walls (Demeyer, 1981; Coughlan, 1985). A complete system for the degradation of cellulose comprises endoglucanases, cellobiohydrolases and β-glucosidases (Coughlan, 1985). The amorphous regions in the cellulose fibre are first attacked by endoglucanases, thereby exposing free chain ends (Fig. 4). Cellobiohydrolases hydrolytically remove cellobiose units from the non-reducing chain ends. The liberated cellobiose is further hydrolysed to glucose by β-glucosidases (Lamed & Bayer, 1988). Endoglucanases and cellobiohydrolases have a strong synergistic action in the degradation of cellulose (Wood & McCrae, 1977).

Hemicelluloses constitute a heterogeneous mixture of homo- and heteroglycans in the plant cell wall (Prins, 1977). The most abundant hemicelluloses are branched-chain β-1-4 xylans and glucomannans (Demeyer, 1981). These polymers may contain varying amounts of other sugars, such as arabinose, galactose, mannose and uronic acids (Prins, 1977; Bisaria & Ghose, 1981). In plant cell walls the hemicelluloses are covalently bound to lignin and are partly esterified with acetic and phenolic acids (Demeyer, 1981). Hemicelluloses are degraded by a variety of enzymes with endo- and exohydrolytic working mechanisms (Hespell, 1988; Wong et al, 1988).

Pectin consists largely of unbranched chains of α-1-4 linked D-galacturonic acid units with many of the carboxyl groups esterified with methyl groups (Bailey, 1973). In the side chains of the pectin molecule galactose, glucose, arabinose, sorbose and xylose residues may occur. Rhamnose residues can be found in the linear part of the molecule (Prins, 1977). Pectin is degraded by pectin esterases, which catalyse the removal of methanol, and depolymerising enzymes, which are either hydrolases or lyases (Prins, 1977).

Bacteria, fungi and protozoa colonise practically all plant materials that enter the rumen (Cheng et al, 1977; Amos & Akin, 1978; Orpin, 1977b). Plant cell walls are degraded and fermented in the rumen by the combined activities of (hemi)cellulolytic and pectinolytic bacteria and to a lesser extent, by protozoa and fungi (Wolin & Miller,1983; Chesson & Forsberg, 1988). Studies with ruminal bacterial strains indicated

that the amount of polysaccharide degrading enzyme activities can be influenced by the growth substrate (Hespell et al, 1987; Williams & Withers, 1982). Substrate preferences and sequential utilization have been found in several bacterial strains from the rumen and are important in the competition between these bacteria (Russell & Baldwin, 1978).

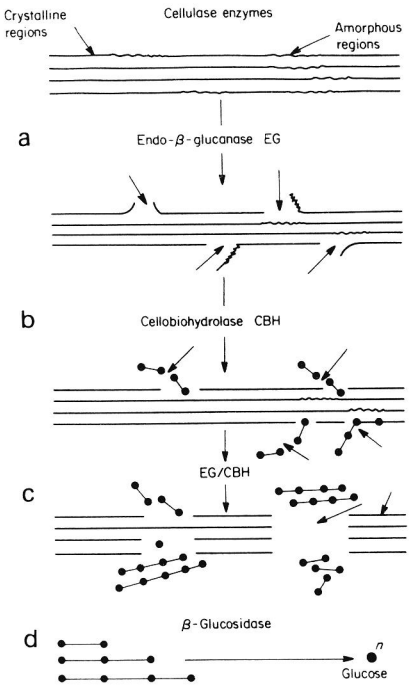


Fig. 4. Schematic representation of synergistic action of enzymes in cellulolysis (Bisaria & Ghose, 1981). Reproduced with permission of Butterworth - Heinemann Publishers.

The main fermentation products of (hemi)cellulose, pectin and starch are volatile fatty acids (acetic, propionic, butyric and C_5-C_7 acids), carbon dioxide and methane (Baldwin, 1984). A typical stoichiometry for the fermentation of glucose, the monosaccharide unit of cellulose and starch, in the rumen is approximately (Hungate, 1966; Wolin, 1979) :

57.5 hexose $\longrightarrow$ 65 acetic acid + 20 propionic acid + 15 butyric acid + 35 CH_4 + 60 CO_2 + 25 H_2O

The proportions of the individual products, however, change with the diet. The major pathway for hexose fermentation of most of the individual species of carbohydrate-fermenting microorganisms in the rumen is the Embden-Meyerhof pathway (Wolin, 1979). Alternative catabolic routes employing the Entner-Douderoff, pentose, and pentose phosphoketolase pathways are also used by many rumen bacteria to metabolise hexoses (Russell & Wallace, 1988). Pyruvate, the central end product of these pathways, is converted to a number of end products, depending on the organisms and growth conditions, with acetic, propionic and butyric acid as major fermentation products (Fig. 5).

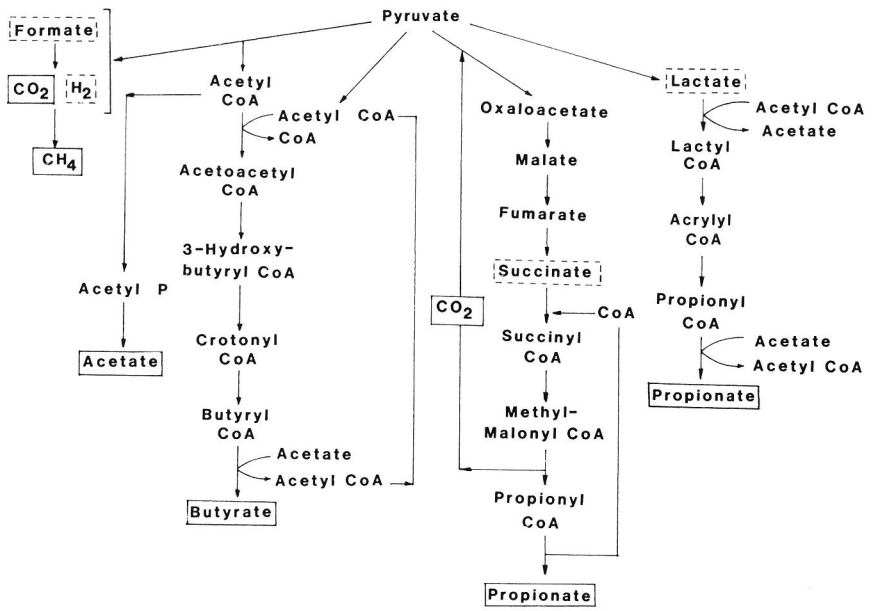


Fig. 5. Metabolic pathways of the degradation of pyruvate to various end-products in the rumen (Lin et al, 1985). Reproduced with permission of Butterworth - Heinemann Publishers.

Feed protein is usually hydrolysed rapidly in the rumen (Wallace & Cotta, 1988). The first step in protein degradation in the rumen is hydrolysis of proteins by proteinases and peptidases. The amino acids produced are either utilized directly by the microflora or degraded further by deaminating enzymes to short chain fatty acids and ammonia (Prins, 1977; Hobson & Wallace, 1982a). Most of the proteolytic activity of rumen fluid is associated with bacteria, whether free-living or attached to plant fibres

(Brock et al, 1982; Kopecny & Wallace, 1982). Ciliated protozoa are of relatively little importance in the breakdown of soluble proteins in rumen contents. These microorganisms are primarily involved in the turnover of bacterial protein in the rumen : they engulf bacteria and excrete amino acids and ammonia (Coleman, 1986; Williams, 1986; Wallace & Cotta, 1988). The contribution of fungal proteinases to the total proteolytic activity in the rumen may also be substantial (Wallace & Joblin, 1985; Wallace & Munro, 1986), but was not studied in detail thus far.

Dietary lipid is degraded extensively in the rumen (Hobson & Wallace, 1982b). Dietary triacylglycerol and phospholipids are hydrolysed in the rumen, yielding glycerol and fatty acids. Glycerol is fermented by rumen microorganisms in a way comparable to carbohydrates (Baldwin, 1984). Lipolytic bacteria, with the potential for degrading the different lipid components of the diet, have been isolated from rumen contents. The role of protozoa in these processes, however, is not clear. Fungi have not, to date, been shown to be able to hydrolyse dietary lipid in the rumen (Harfoot & Hazlewood, 1988). Unsaturated fatty acids released are extensively hydrogenated by the microbes forming *trans* monoenoic and saturated fatty acids (Hazlewood et al, 1976; Kellens et al, 1986; Harfoot & Hazlewood, 1988). Fat not hydrolysed in the rumen is digested in the lower parts of the gut (Leat & Harrison, 1975).

Interactions between microorganisms

Plant polysaccharides are the primary carbon and energy sources for rumen microorganisms. Although the cell wall degrading enzymes, needed to degrade these polysaccharides are limited to only a few species of bacteria, protozoa and anaerobic fungi, hydrolysis products of these polysaccharides support the growth of many species incapable to use these substrates directly. The latter species successfully compete with the polymer degrading species for the products of hydrolysis (Wolin & Miller, 1988). Another way for non-hydrolytic species to survive in the rumen is to use the fermentation products of hydrolytic species like succinate, lactic acid, formate and H_2.

Succinate is an important extracellular intermediate in the overall rumen fermentation. It is decarboxylated as fast as it is formed by propionic acid producing bacteria like *Selenomonas ruminantium*. The extracellular pool of succinate is extremely small (Wolin, 1975; 1979). Lactic acid is produced when animals are fed considerable quantities of easily degradable, starch-containing diets. Lactic acid fermenting species, like *Megasphaera elsdenii* and *Veillonella alcalescens*, are able to use this compound as an energy source (Wolin, 1979). Formate does not accumulate in the rumen since it is converted rapidly to methane (Wolin, 1979). The same applies to hydrogen : essentially all the hydrogen produced in the rumen is used immediately by rumen methanogens to reduce CO_2 to CH_4 (Demeyer & Van Nevel, 1975; Wolin & Miller, 1988). Interspecies hydrogen transfer and the environmental pressure of hydrogen are probably the most important factors regulating the microbial fermentation in the rumen (Wolin, 1979). Due to the substantial capacity of rumen methanogens to use hydrogen the partial hydrogen pressure in the rumen is 10^{-4} atm or less (Hungate, 1966). Since interspecies hydrogen transfer efficiently reduces the H_2 concentration, rumen microorganisms are

no longer obliged to recycle electrons generated during glycolysis to carbon compounds but are free to produce H_2 from NADH and thereby generate more ATP per mole of substrate (Thauer et al, 1977). Thus the electron flow shifts away from use in reductive reactions such as the formation of ethanol to more oxidized products as acetic acid (Wolin, 1979).

Rumen metabolism can be altered by changes in the diet. Chemical or physical treatments can be used to increase the degradability of crude fibre in the rumen and feed protein can be protected against microbial attack (Van Nevel & Demeyer, 1988). The fermentation pattern can also be changed by the use of certain compounds, which act on groups of microorganisms. Many chemicals can inhibit methanogenesis and enhance the production of propionic acid and, in some cases, butyric acid (Demeyer & Van Nevel, 1975). Since the production of methane represents an energy loss to the animal, more energy will be available when methanogenesis is inhibited. Ionophore antibiotics like monensin, which enhance propionic acid production and reduce methane production, are used as major feed additives (Chen & Wolin, 1979). A better performance of cattle and sheep on all kinds of diets is seen when monensin is used (Van Nevel & Demeyer, 1988). The increase in propionic acid production results in a protein-sparing effect since less amino acids are used for gluconeogenesis under these circumstances (Schelling, 1984).

Influence of the diet

The ecology of the rumen microorganisms is very complex. Several microbial species present can ferment various classes of carbohydrates and are present in greater or smaller numbers on all diets. However, not all microorganisms do compete equally well for all substrates, so the proportions of each class of carbohydrates in various feeds can cause large shifts in the relative numbers of the many microbial species in the rumen (Baldwin, 1984). These changes in the microbial population influence product formation in the rumen (Hobson, 1988). Changing the diet from high roughage to high concentrate results in a rise in the number of amylolytic and acid-tolerant bacteria and a decrease of the number of the cellulolytic bacteria (Kaufmann et al, 1980). The proportions of the individual VFA also change with the diet. The proportion of propionic acid is higher with concentrate diets. In general, amylolytic microorganisms produce considerably more propionic acid than cellulolytic microorganisms, whereas saccharolytic and pectinolytic microbes are intermediate. Roughage rations are high in cellulose, intermediate in soluble sugars and low in starch. Therefore, numbers of cellulolytic and saccharolytic microorganisms are high, and large proportions of acetic acid are formed, not only due to cellulose fermentation but also because the saccharolytic microorganisms compete favourably for soluble carbohydrates and the hydrolytic products of starch and hemicellulose hydrolysis. Thus, propionic acid production is increased when cereals are fed, not only because of the high starch fermentation, but also because the fermentation products formed from other carbohydrates are altered to favour propionic acid (Baldwin, 1984). However, acetic acid is always the most abundant product (Wolin, 1979; Baldwin, 1984). The most important

adaptation process to changes in dietary composition is the pH regulation system. The interaction between feed structure, saliva production and rumination allows the establishment of a pH, at which degradation of dietary components is most effective (Kaufmann et al, 1980).

Gonzalez-Lopez et al (1990) showed considerable changes in the composition of the bacterial flora in rumen fluid *in vitro* depending on the substrate. The addition of wheat straw, a substrate high in crude fibre, resulted in the increase of cellulolytic bacteria, particularly *Bacteroides fibrisolvens*. When alfalfa hay was added to rumen fluid an increase in the number of bacteria with a marked proteolytic activity was noticed (Gonzalez-Lopez et al, 1990).

Detoxification in the rumen

Ruminant animals are more resistant to the effect of many toxins compared to monogastric animals. This resistance can often be related to the conversion of these compounds by rumen microorganisms (Dawson & Allison, 1988). Hydrolytic, reduction and fission reactions are the main reactions involved in the metabolism of toxic compounds in the rumen (Prins, 1987). Toxins in plant feed material, such as oxalate, pyrrolizidine alkaloids, nitrate (nitrite) and mimosine are detoxified by rumen microorganisms (Dawson & Allison, 1988). Ruminant animals are able to acquire tolerance to increased concentrations of toxic materials in their feeds. In some cases this acquired tolerance can be related to changes in populations of rumen microorganisms leading to increased rates of toxin degradation (Dawson & Allison, 1988).

Mimosine [β-(3-hydroxy-4-oxopyridyl) α-amino-propionic acid] is a toxic amino acid found in tropical leguminous shrubs belonging to the genus *Leucaena*. In the rumen mimosine is converted to 3-hydroxy-4-(1H)-pyridone (3,4-DHP), a potent goitrogen, and to 3-hydroxy-2-(1H)-pyridone (2,3-DHP) (Hegarty et al, 1976). These dihydroxypyridines (DHP) can be degraded by bacteria present in the rumina of animals in some parts of the world (Allison et al, 1990). Apparently, the DHP-degrading microorganisms are indigenous in the rumen microflora in these parts of the world, and are present even when *Leucaena* is not a part of the diet (Dominguez-Bello & Stewart, 1990a). In other areas these DHP-degraders were not found in the rumen of cattle, sheep and goats and these animals exhibit *Leucaena* toxicosis when fed *Leucaena* plants (Allison et al, 1990). DHP-degrading microorganisms can be successfully transferred to the rumina of animals not able to degrade DHP (Jones & Lowry, 1984; Jones & Megarrity, 1986; Hammond et al, 1989).

Another toxic amino acid, canavanine, can also be degraded by a range of rumen bacteria (Dominguez-Bello & Stewart, 1990b).

A number of toxic compounds is present in rapeseed or soybeans or formed upon handling these feed ingredients. They will be dealt with in the next paragraph.

Soybeans and rapeseed

Soybeans and rapeseed are both major sources of edible fats and oils (Pryde & Doty, 1981). The meal remaining after the extraction of oil is used as a protein feed for livestock.

The oil is currently extracted by pressing and/or solvent extraction. These processes require a high capital investment in solvent recovery equipment and the use of highly inflammable solvents increases the risk of the process. The organic solvents used in the extraction processes can also cause environmental problems. The use of enzymes in oil extraction reduces these risks and these problems (Godfrey, 1983). Additional advantages of the use of enzymes in oil extraction processes are an improvement of the oil yield, a lower oil content in the final seed cake feedstuffs, reduction in the acid development and oxidation of the oil during further processing and storage, removal of toxins from the oilseed protein by the aqueous processing and a good oil quality (Fullbrook, 1983; Godfrey, 1983; Szakács-Dobozi et al, 1988).

The protein rich meal is an important coproduct of the oil extraction from soybeans and rapeseed and is used extensively as a protein feedingstuff for pigs, poultry and ruminants. However, meals resulting from both seeds contain antinutritional compounds : soybean meal contains protease inhibitors, rapeseed meal contains glucosinolates, tannins and erucic acid (Brooks & Morr, 1984; Fenwick, 1984; Sessa & Bietz, 1984; Daun, 1986a).

The most limiting factor for the use of rapeseed meal as a food seems to be the presence of glucosinolates (Josefsson, 1972). The general structure of glucosinolates is given in Fig. 6 (Fenwick et al, 1983).

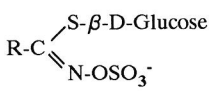


Fig. 6. The general structure of glucosinolates.

Nearly all glucosinolates known (about 90) possess the same basic skeleton. Differences between glucosinolates depend on the chemical nature of the side chain (R) and this also has a significant effect upon the ultimate products of hydrolysis (Fenwick et al, 1983; Fenwick & Heaney, 1983). The majority of these glucosinolates (74) is listed by Ettlinger & Kjaer (1968). Glucosinolates are hydrolysed when the wet, unheated plant material is crushed, for instance by fretting. The hydrolysis is catalysed by myrosinase, an enzyme (or group of enzymes) which behaves as a thioglucosidase (Fenwick et al, 1983). This enzyme is present in the plant material (VanEtten et al, 1969). When glucosinolates are hydrolysed by myrosinase isothiocyanates, thiocyanates and nitriles are formed, their formation depending on factors as the structure of the

glucosinolate per se, pH and the presence of compounds like ascorbic acid or ferrous ions, which modify the action of the enzyme (Fenwick et al, 1983).

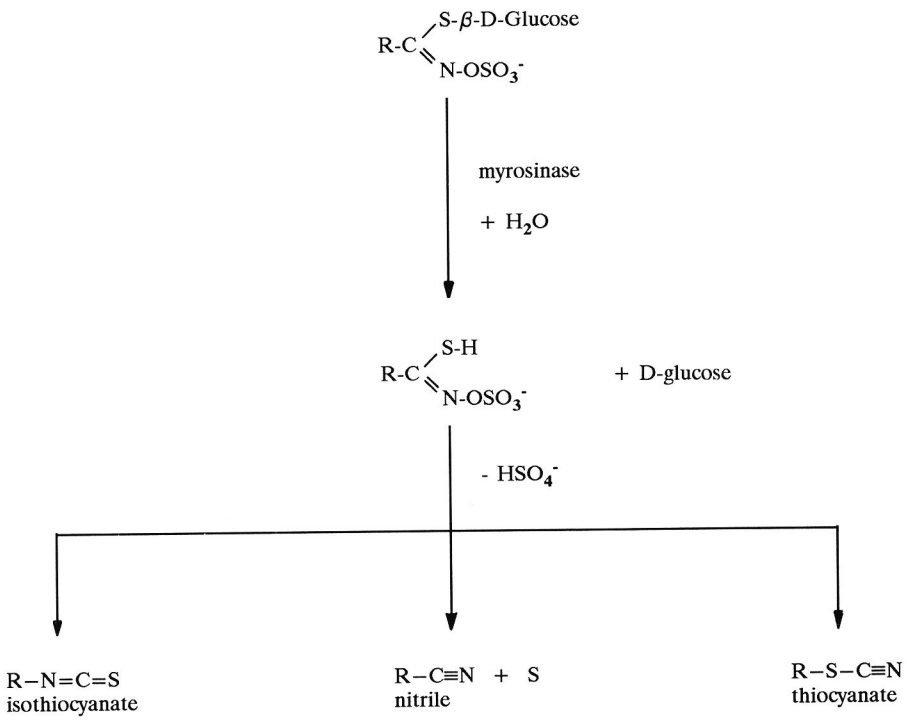


Fig. 7. Products of enzymatic hydrolysis of glucosinolates (VanEtten et al, 1969; Fenwick et al, 1983).

In rapeseed several glucosinolates are encountered (Josefsson, 1972). The toxic hydrolysis products of these glucosinolates are responsible for the observed antinutritional effects. Ruminants seem to have a higher resistance to these effects than pigs and poultry (Rutkowski, 1971; Bell, 1984).

Outline of this thesis

Recently, an artificial rumen system was developed resembling well the *in vivo* rumen system (Gijzen et al, 1986). Further studies concentrated on the use of this artificial rumen system for the degradation of organic waste materials. This thesis deals

with the study of biotechnological applications of this system in food industry : the prediction of rumen digestibility of dietary ingredients, the induction of "tailor-made" enzyme systems, specifically suited to degrade soybean and rapeseed cell walls, the induction of different cellulolytic enzymes by loading the system with different substrates and the use of the system as a model to study the metabolism of (toxic) compounds in the rumen.

General methods will be described in Chapter 2.

In the artificial rumen reactor high loading rates can be tested without problems concerning palatability or animal toxicity. In Chapter 3 the system will be used to test the effect of specific ingredients of the ruminal diet. The digestibility of several soybean and rapeseed products will be measured at various loading rates under standardized conditions *in vitro*.

The cellulolytic capacity of an artificial rumen system fed pure cellulose will be studied in Chapter 4. This capacity will be compared with the cellulolytic potential of fractions of the system to determine the possible effect of the treatments applied to obtain these fractions.

In Chapter 5 degradation efficiencies and specific enzyme levels in artificial rumen reactors loaded with filter paper cellulose, low protein soybean meal and low protein rapeseed meal will be measured. As the chemical composition of these substrates shows marked differences possible changes in the activity pattern of (hemi)cellulolytic enzymes induced by the diet will be studied.

Cellulolytic enzymes can be applied to enhance the extractability of the oil and to improve the oil yield from soybeans and rapeseed. The use of fractions from artificial rumen systems for this purpose will be described in Chapter 6. The efficiency of fractions of artificial rumen systems loaded with low protein soybean meal, low protein rapeseed meal and filter paper cellulose in improving the oil yield from soybeans and rapeseed will be tested. The oil yield obtained by these fractions will be compared to yields obtained when a commercial enzyme preparation (Viscozyme^{TM}L, NOVO) or the extracellular enzymes of an anaerobic fungus (*Piromyces* spec.) are used.

Ruminants are usually less susceptible for the presence of antinutritional compounds or toxins in their diet than monogastric animals, such as poultry and pigs. The fate of certain antinutritional compounds in the rumen will be studied in Chapter 7. The effect of a toxic hydrolysis product from glucosinolates in rapeseed, L-5-vinyl-oxazolidine-2-thione (VOT), on rumen performance will be tested to explain the observed resistance of ruminants to the inclusion of rapeseed meal in their diet.

2

GENERAL METHODS

Fermenter design and operational conditions

The *in vitro* fermentations were carried out in an "artificial rumen" reactor with a 1.5 l working volume and operating with differential removal rates of solids and liquids as described earlier (Gijzen et al, 1986). A schematic diagram of the reactor is shown in Fig. 1.

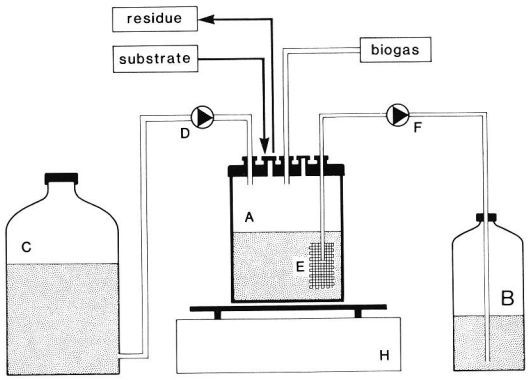


Fig. 1. Schematic diagram of the artificial rumen reactor.
A, 3 litre cylindrical glass vessel; B, filter effluent reservoir; C, buffer vessel; D, F, peristaltic pumps; E, cylindrical filter (30 μm pore size); H, laboratory rotary shaker, containing up to four reactors.

Briefly, reactors were kept at 39°C and inoculated with 250 ml of strained rumen fluid obtained from a fistulated sheep. After inoculation reactors were loaded with a mix of alfalfa and the different soybean and rapeseed substrates, starting with 50 % alfalfa in order to gradually change the dietary composition. During the first week of operation the amount of alfalfa was gradually decreased to zero. The reactor receiving filter paper cellulose (FPC) was fed twice the normal loading rate on the day of inoculation. The FPC was supplemented with 2 $g.l^{-1}.d^{-1}$ ground alfalfa hay as an additional nutrient source. Digester feed was added once every day.

Mineral buffer according to Rufener et al (1963), supplemented with trace elements (0.2 $ml.l^{-1}$) according to Vishniac & Santer (1957) was added continuously to the reactor at a rate adjustable by a peristaltic pump. Except for the day of inoculation, the complete buffer was supplemented with 28 mM NH_4HCO_3 for cultures loaded with FPC. No ammonium salts were included in the mineral buffer when soybean or rapeseed substrates were used. Liquid fermentation medium was continuously removed through a filter unit. Homogeneous reactor content (600 ml) was removed once daily

before addition of the substrate, resulting in a solid retention time of 72 h. Different solid- and liquid- retention times were established in this way. The reactor contents were mixed every 15 min for a period of 30 s. When reactor conditions were changed an acclimatization period of 5 - 7 days was followed by an experimental period of 7 days. Sampling was performed as described previously (Gijzen et al, 1986).

Digester feeds

Soybean flakes and defatted soybean flakes were purchased from Central Soy (Utrecht, the Netherlands), rapeseed and defatted rapeseed (low glucosinolate quality) from Speelmans oliefabriek (Rotterdam, the Netherlands), alfalfa hay (*Medicago sativa*) from van Heeswijk (Veghel, the Netherlands). Soybean flakes, soybean meal and rapeseed meal were used as such, rapeseed was frozen in liquid nitrogen and milled in a coffee grinder. Whatman filter paper cellulose grade 91 was used as a pure cellulose substrate. The filter paper cellulose was reduced to a particle size of 5 to 10 mm.

To remove part of the protein soybean meal and rapeseed meal were milled to pass a 0.2 mm sieve and treated in a 0.1 M NaCl solution (pH 8.5) for two hours at room temperature. The salt solution was replaced once during that time, pH was checked regularly and adjusted when necessary. After washing with tap water the residual material was dried at 70°C for 16 h. This treatment resulted in the removal of approximately 0.05 g and 0.15 g protein per g dry weight for rapeseed meal and soybean meal, respectively.

The chemical composition of the substrates is listed in Table 1. The protein content of untreated rapeseed and soybean meal amounts to approximately 40 and 50 % of the dry matter, respectively (Josefsson, 1972).

Analytical methods

Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were estimated according to Goering & van Soest (1970).
Analyses for total solids (TS) and volatile solids (VS) were carried out according to standard methods (Anon., 1975). Cell solubles (CS) were calculated as VS minus NDF (Chandler et al, 1980). Fermentation products were analyzed on a Hewlett Packard 5890 A gas chromatograph equipped with a flame ionisation detector according to Teunissen et al (1989).

The protein content of reactor samples was determined after extraction in 0.5 M NaOH for three days at room temperature with daily vortexing (McKinley & Vestal, 1985). The sample was centrifuged, the obtained supernatant was diluted as appropriate and the protein content of these samples was determined in triplicate according to Bradford (1976) with bovine serum albumin (Serva) as a standard.

Methane was measured as described by Hutten et al (1981).

All other analyses and calculations were performed as described previously (Gijzen et al, 1986; 1987).

Table 1. Chemical composition of the substrates

Substrate	TS (%)	VS (% of TS)	NDF (% of TS)	ADF (% of TS)	CS (% of TS)
SOYBEAN					
Flakes	92.7 ± 0.3[a]	83.4 ± 2.7	11.0 ± 1.7	6.7 ± 0.6	2.4 ± 11.4
SM	87.8 ± 2.0	82.6 ± 2.4	16.6 ± 1.3	11.0 ± 1.0	66.0 ± 5.5
LPSM	93.1 ± 1.7	89.8 ± 7.5	43.4 ± 6.3	25.6 ± 6.0	46.4 ± 4.4
RAPESEED					
Seed	93.7 ± 1.7	83.6 ± 1.9	n.d.[b]	n.d.	n.d.
RM	91.0 ± 2.5	91.2 ± 3.5	26.7 ± 2.7	22.9 ± 1.6	64.5 ± 10.8
LPRM	90.3 ± 1.4	83.9 ± 2.6	37.8 ± 2.3	26.4 ± 2.7	46.1 ± 3.1
FPC	93.9 ± 0.1[c]	99.8 ± 0.1	98.0 ± 1.0	98.0 ± 1.0	1.3
Alfalfa	92.3 ± 0.6[c]	87.4 ± 0.9	43.8 ± 0.2	31.5 ± 0.7	43.6 ± 0.6

Abbreviations : TS total solids; VS volatile solids; NDF neutral detergent fibre; ADF acid detergent fibre; CS cell solubles; SM soybean meal; LPSM low protein soybean meal; RM rapeseed meal; LPRM low protein rapeseed meal; FPC filter paper cellulose

[a] All values are expressed as means ± SD, for n = 3 - 12

[b] Not determined (high amount of oil disturbs analysis)

[c] Data from Gijzen et al (1988)

Substrates for the measurement of polysaccharidase-activities

Carboxymethylcellulose, a soluble form of cellulose, consists of linear chains of β-1-4 linked glucose residues. Avicel, a micro-crystalline form of cellulose, also consists of linear chains of β-1-4 linked glucose residues. The glucan chains in this polymer aggregate to microscopically visible fibrils, in which the individual glucan chains are cross-linked by hydrogen bonding. In filter paper cellulose, the third type of cellulose used, the degree of cross-linking even is more extensive.

Glycogen consists of chains of α-1-4 linked glucose residues, which are cross linked approximately every 8^{th} glucose residue by an α-1-6 linkage.

Xylan, a hemicellulosic polymer, is based on β-1-4 linked chains of xylopyranosyl units, carrying side chains of single glucuronic acid units and short branched chains of α-1-3 and α-1-5 linked arabinofuranose residues.

Pectin is based on chains of α-1-4 linked galacturonic acid units,in which the carboxylic acid groups are variably esterified with methanol and the uronic acid residues variably substituted at carbon-2 with acetyl groups. Rhamnose units are found throughout the strain, linked 1-2 to adjacent uronic acid residues.

Locust bean gum, a galactomannan, consisting of a chain of α-1-4 linked mannose residues to which single α-D-galactosyl residues are attached.

Arabinogalactan, a hemicellulosic polymer, consists of β-1-3 linked galactose residues, carrying side chains of arabinose and galactose residues.

Determination of total (hemi)cellulolytic activity

To determine total cellulase activities in artificial rumen samples, cells were broken by ultrasonic disintegration (Branson B12, Danburg, Connecticut) at 4°C in the presence of glass beads (Ø 4 mm) in a volume of 25 ml. After centrifugation (48,000 x g , 30 min, 4°C) the pellet was resuspended in the original volume of demineralised water and the procedure was repeated twice. Polysaccharidase activity was determined in all supernatant fractions by measuring the amount of reducing sugars released from the polysaccharide substrate (0.5 % in 0.1 M Na-acetate buffer, pH 5.5). Carboxymethylcellulose (Sigma) and avicel (Serva) were used to estimate cellulolytic activities, oat spelt xylan (Sigma) to estimate hemicellulolytic activities in the different samples. Xylan was washed three times in demineralised water and lyophilised before use. Incubations were performed at 39°C for 20 min (carboxymethylcellulose, xylan) or 120 min (avicel). The amount of reducing sugars was determined according to the procedure of Nelson and Somogyi (Somogyi, 1952) with glucose or xylose as a standard as appropriate.

β-glucosidase activities were determined by measuring the release of p-nitrophenol from p-nitrophenyl-β-D-glucopyranoside (5 mM in 50 mM Na-citrate buffer, pH 4.8). The reaction was stopped by the addition of one volume of 2 M Na_2CO_3. After dilution with 4 ml demineralised water the release of p-nitrophenol was determined spectrophotometrically at 412 nm with p-nitrophenol as a standard.

Chemicals

Chemicals used for the mineral buffer were of technical grade. Trace elements and all other chemicals used were of analytical grade.

APPLICATION OF AN *IN VITRO* ARTIFICIAL RUMEN SYSTEM TO TEST THE DIGESTIBILITY OF SOYBEAN AND RAPESEED MEAL

SUMMARY

An artificial rumen system was used as an *in vitro* test system for the digestion of plant raw materials. Different pretreatments of soybeans and rapeseed and various loading rates were applied. Both soybean and rapeseed substrates were degraded in the artificial rumen, however the latter substrates were degraded less efficiently. The oil present in soybean flakes and rapeseed had a marked negative effect on the functioning of the system. Fermentation product ratios from the *in vitro* system were comparable to those from feeding trials *in vivo*. The artificial rumen system can be used to predict the digestibility of plant raw materials used as feed components in ruminal diets.

INTRODUCTION

Soybean meal occupies a leading position in the world production of oilseed meals. Rapeseed meal production is significantly lower but also important as a source of feed protein (Rutkowski, 1971). The protein content of soybean meal and of rapeseed meal amounts to approximately 50 % and 40 % of the dry matter, respectively (Josefsson, 1972). The amino acid composition of soybean and rapeseed meal is quite similar : rapeseed meal contains somewhat more S-containing amino acids and less lysine. These meals, which remain after the extraction of oil from the oilseeds, represent a product of an equal economic value to the oil itself for the oilseed industry. The meals are normally used as a protein feed for livestock. The fibre content of rapeseed meal is about twice as high as the fibre content of soybean meal and this results in a reduction in energy value and the bio-availability of minerals.

In addition, the use of oilseed meals as animal food is limited because of the presence of antinutritional compounds or their precursors, such as glucosinolates, tannins and erucic acid. This problem has been partly overcome by breeding cultivars low in glucosinolates and / or erucic acid (Fenwick, 1984). Difficulties with the palatability of rapeseed meal were most frequently encountered with sheep, cattle and pigs and result from the formation of pungent products from glucosinolates. Rapeseed meal low in glucosinolates appeared to be much more palatable to swine and cattle than the high glucosinolate cultivars (Fenwick, 1984).

The digestion of soybean meal and rapeseed meal in the rumen has always been tested in feeding trials with cattle (Lindell, 1976; Fisher & Walsh, 1976; Rooke et al, 1982; Arambel et al, 1986). In these *in vivo* trials soybean meal and rapeseed meal were tested as portions (up to 35 %) of the concentrate part of the diet. Reliable tests of rumen digestibility of oilseed meals *in vitro* are lacking.

Recently, Gijzen et al (1986) developed an artificial rumen system resembling well the *in vivo* rumen, when fed with animal feed. The present study reports on the use of this artificial rumen system to test the influence of several soybean and rapeseed substrates on rumen performance, in order to assess the effect of single ingredients of the diet.

RESULTS

Digestion of the substrates in the artificial rumen reactor

Degradation efficiency of soybean and rapeseed substrates was studied at different loading rates with a solid retention time of 72 h and a hydraulic retention time of 14 ± 1.5 h. A steady-state with respect to degradation and fermentation parameters was reached in about 14 days. When the operational conditions were changed an acclimatization period of 7 days was used. Efficiency of degradation, expressed in parameters, such as neutral detergent fibre (NDF) and volatile solids (VS) degradation, pH, volatile fatty acids (VFA) and biogas production and specific VFA and biogas production are shown in Table 1, 2 and 3.

Table 1. Steady state degradation of the different substrates, pH and production of fermentation products in the artificial rumen reactor

Substrate	LR (g VS. $l^{-1}.d^{-1}$)	pH	Degradation (%) NDF	VS	Production VFA ($mmol.l^{-1}.d^{-1}$)	Biogas[a] ($l.l^{-1}.d^{-1}$)
SOYBEAN						
Flakes	21	6.8 ± 0.1[b]	77	51	79 ± 11[b]	1.5 ± 0.4[b]
SM	19	6.9 ± 0.1	92	83	139 ± 12	3.5 ± 0.4
	25	6.8 ± 0.1	96	90	135 ± 5	4.0 ± 0.6
	30	6.7 ± 0.1	91	91	147 ± 11	5.1 ± 0.7
	34	6.7 ± 0.1	90	90	160 ± 12	5.7 ± 0.1
LPSM	22	6.7 ± 0.1	96	85	156 ± 6	3.0 ± 0.4
	28	6.7 ± 0.1	95	86	139 ± 7	4.7 ± 0.3
	32	6.6 ± 0.1	95	85	150 ± 13	5.5 ± 0.3
	37	6.4 ± 0.1	94	82	142 ± 2	5.5 ± 0.5
	42	6.2 ± 0.1	93	84	152 ± 6	6.3 ± 0.3
	46	6.1 ± 0.2	93	74	141 ± 12	6.1 ± 0.8
	69[c]	6.3 ± 0.1	90	76	233 ± 2	8.7 ± 0.5
RAPESEED						
Seed	21	6.7 ± 0.1	n.d.[d]	50	79 ± 9	0.9 ± 0.2
RM	29	6.6 ± 0.1	55	60	118 ± 9	2.8 ± 0.5
LPRM	28	6.7 ± 0.1	62	64	110 ± 7	2.6 ± 0.2

Abbreviations : LR loading rate, SM soybean meal, LPSM low protein soybean meal, RM rapeseed meal, LPRM low protein rapeseed meal

[a] Mean methane content of the biogas 20 ± 5 % ; for rapeseed and soybean flakes 5 ± 5 %

[b] Means ± SD, n = 3 - 5

[c] Buffer supplemented with 2.4 g $NaHCO_3.l^{-1}$

[d] Not determined (high amount of oil disturbs analysis)

Table 2. Specific productions of VFA and biogas during steady state fermentation

Substrate	LR ($g\ VS.l^{-1}.d^{-1}$)	VFA production ($mmol.g^{-1}$ VS digested)	Biogas production ($l.g^{-1}$ VS digested)
SOYBEAN			
Flakes	21	7.5 ± 1.0[a]	0.14 ± 0.04[a]
SM	19	8.6 ± 0.7	0.22 ± 0.03
	25	5.9 ± 0.2	0.18 ± 0.03
	30	5.5 ± 0.4	0.19 ± 0.03
	34	5.3 ± 0.4	0.19 ± 0.01
LPSM	22	8.2 ± 0.3	0.16 ± 0.02
	28	5.8 ± 0.3	0.20 ± 0.01
	32	5.5 ± 0.5	0.20 ± 0.01
	37	4.7 ± 0.1	0.18 ± 0.02
	42	4.5 ± 0.2	0.18 ± 0.01
	46	4.2 ± 0.4	0.18 ± 0.02
	69	4.4 ± 0.1	0.17 ± 0.01
RAPESEED			
Seed	21	6.8 ± 0.8	0.08 ± 0.02
RM	29	5.8 ± 0.4	0.14 ± 0.03
LPRM	28	5.6 ± 0.4	0.13 ± 0.01

[a] Means ± SD, n = 3 - 5

Table 3. Mean molar ratio of VFA (%), produced during steady-state degradation of different substrates at various LR

Substrate	A	P	IB	B	IV	V
SOYBEAN						
Flakes	53	31	1	9	3	3
SM	52	19	4	15	6	4
LPSM	54	21	3	11	6	4
RAPESEED						
Seed	40	44	1	7	4	5
RM	48	28	1	12	4	8
LPRM	57	26	1	9	4	4

Abbreviations : A acetic acid, P propionic acid, IB isobutyric acid, B butyric acid, IV isovaleric acid, V valeric acid

Effect of the substrate

Rapeseed and soybean flakes contain 40 % and 20 % oil (w/w), respectively. This oil had a considerable effect on the functioning of the reactor : VS-degradation did not exceed 50 %, whereas VS-degradation varied from 70 - 90 % when substrates without oil were used. Biogas- and VFA-production were significantly higher when the system was loaded with defatted substrates. Loading the reactor with soybean flakes or rapeseed yielded relatively high proportions of propionic acid. The degradation of defatted and low protein substrates gave rise to slightly higher amounts of acetic, butyric, isovaleric and valeric acid.

Table 4. Ciliated protozoa numbers and species composition during steady state fermentation of SM and LPSM

Substrate	Total number (10^3.ml^{-1})	Ciliated protozoa (% of total)			
		Diplodinia	*Epidinia*	*Entodinia*	*Holotrichs*
SM[a]	10 ± 2	17 ± 9	2 ± 1	81 ± 9	0
LPSM[b]	8 ± 3	44 ± 8	1 ± 1	55 ± 8	0
Inoculum	560	7	2	84	9

[a] LR 19 g VS.l^{-1}.d^{-1}
[b] LR 22 g VS.l^{-1}.d^{-1}

Total ciliated protozoa numbers were low and members of the *Entodinium*-group were predominant, during steady state degradation of the different substrates (Table 4). When soybean flakes or rapeseed were used as substrates, ciliated protozoa numbers decreased rapidly and within 5 and 14 days after inoculation, respectively, all ciliated protozoa had disappeared from the system. With RM and LPRM *Entodinia* were the predominant species as with the soybean substrates (results not shown).

Effect of loading rate (LR)

The effect of the loading rate was studied by applying different loading rates of soybean meal and low protein soybean meal (LPSM) with constant solid and hydraulic retention times (Table 1 and 2). Degradation efficiencies did not differ much at all loading rates applied : when the loading rate of LPSM was tripled VS-degradation was still 76 %. Fermenter pH decreased at these higher loading rates; to prevent pH decrease below 5.7 extra bicarbonate was added at the highest loading rate. Biogas-

production increased with increasing loading rate but the specific biogas-production (0.18 $l.g^{-1}$ VS digested) was constant, not dependent on the loading rate. Specific VFA-production gradually decreased. Loading rates higher than 69 g $VS.l^{-1}.d^{-1}$ LPSM could not be tested since the very high content of solids in the reactor caused mixing problems.

DISCUSSION

The effect of soybean meal or rapeseed meal supplementation to cattle rations is usually tested *in vivo* (Fisher & Walsh, 1976; Thomke, 1981). It is often difficult to compare the influence of these meals on rumen parameters, such as VS and NDF degradation, VFA formation and pH, because of the use of different diets, in which the meals are included when working with animals *in vivo*. On the contrary, *in vitro*, rumen conditions are more standardized, often are better reproducible (Gijzen et al, 1986) and possess a predictive value for the *in vivo* system. An *in vitro* rumen system allows the use of specific hydraulic and solid retention times to study for example the digestibility or other effects of these oilseed meals. Under these circumstances the oilseed meals can be tested as the sole substrate for the artificial rumen reactor. High loading rates of these substrates may also be used without danger of animal toxicity.

Soybean and rapeseed substrates can be typed as materials rich in cell solubles (CS). CS-rich materials may be considered as readily digestible substrates because the CS fraction includes easily degradable compounds such as soluble sugars, pectin and starch (Gijzen et al, 1987). It has been shown that the protein of soybean meal and rapeseed meal is also readily degradable in the rumen. Varvikko (1986) reported a 70 % breakdown of soy protein and a 78 % breakdown of rapeseed protein *in sacco* in the rumen. Our results also show that the degradation efficiency was high when soybean meal or low protein soybean meal was used as a substrate in the artificial rumen system. However, when soybean flakes were used a marked effect of the oil present in the substrate on NDF- and VS-degradation and ciliated protozoa composition was observed. This has also been shown by Macleod & Buchanan-Smith (1972) and Ikwuegbu & Sutton (1982), who reported a reduction in organic matter digestion in the rumen when soybean oil was included in the diet. Doreau & Michalet-Doreau (1988) reported a decrease in the rate of dry matter and NDF-degradation when rations for ruminants were supplemented with fat. A decrease in VS-degradation was obvious when full fat substrates were used as a substrate for the artificial rumen system.

Ciliated protozoa, marker organisms of a well functioning artificial rumen system, disappeared from the reactor when soybean flakes or rapeseed were used as the sole substrate. Total ciliated protozoa numbers were low in reactors loaded with defatted substrates and representatives of the *Entodinium*-group were predominant (Table 4) as was shown before for CS-rich waste materials (Gijzen et al, 1987; Lubberding et al, 1988). Reduction of the number of ciliated protozoa following the addition of linseed-, soybean- or safflower- oil to the rumen as a part of the diet was described by Ikwuegbu & Sutton (1982), Broudiscou et al (1988) and O'Kelly et al (1990). In our experiments

all ciliated protozoa disappeared from the system upon the addition of oil-containing substrates.

In the artificial rumen system rapeseed meal and low protein rapeseed meal were degraded less efficiently (60 - 65 %) compared to soybean meal substrates (80 - 90 %). This may be due to the higher fibre content of rapeseed meal compared to soybean meal. Feeding trials also revealed a lower metabolizable energy value compared to soybean meal (Papas et al, 1978). From these *in vivo* trials it became clear that the amount of rapeseed meal that can be included in the concentrate part of the diet without adverse effects on feed intake, milk yield and composition and general health of the animal is usually limited to 20 - 30 % (Ingalls & Sharma, 1975; Fisher & Walsh, 1976; Papas et al, 1978).

Ingalls & Sharma (1975) included 10 % soybean meal or rapeseed meal in dairy rations and found no differences in dry matter digestibility. Fisher & Walsh (1976), however, found a decrease in digestibility of rapeseed meal (34 % of the concentrate part of the diet) in comparison to soybean meal (27 % of the concentrate part of the diet) in feeding trials with Holstein cows. Results from digestibility tests of feed ingredients in the artificial rumen system thus correspond with the digestibility of these feed ingredients in the rumen in feeding trials *in vivo*. The artificial rumen may be used to predict the digestibility of feed components *in vitro*.

Total VFA-production was influenced by the substrate : on soybean substrates VFA-production was higher than on rapeseed substrates. The relative proportions of individual VFA were typical for the fermentation of CS-rich substrates (Gijzen et al, 1987). Similar relative proportions of VFA were found when an artificial rumen system was loaded with onion pulp, horticultural waste and vegetable auction waste, waste materials rich in cell solubles (Gijzen et al, 1987; Lubberding et al, 1988). Ikwuegbu & Sutton (1982) reported a linear decrease in the molar proportion of acetic and butyric acid when increasing amounts of linseed oil were added to the diet. This coincided with a linear increase in the proportion of propionic acid. When reactors were loaded with full fat substrates a similar increase in propionic acid formation was found. For feeding trials with rapeseed meal and soybean meal VFA-ratios comparable to the ratios reported here were described previously (Ingalls & Sharma, 1975; Sharma et al 1977; Arambel et al, 1986). This VFA ratio is typical for a concentrate-containing diet (Oshio et al, 1987; Lees et al, 1990).

Specific biogas production was constant with increasing loading rate but the specific VFA production gradually decreased to a stable level possibly due to the formation of products not measured here (Table 2). Sniffen et al (1985) reported a significant peptide accumulation in the rumen during the breakdown of soybean meal.

In conclusion, the *in vitro* rumen system can be used to measure the digestibility of plant raw materials, used as ingredients for ruminant feeds, and to predict the effect of these materials in the rumen *in vivo*.

DEGRADATION OF FILTER PAPER CELLULOSE IN THE ARTIFICIAL RUMEN SYSTEM AND MEASUREMENT OF CELLULOLYTIC ACTIVITIES

SUMMARY

An artificial rumen reactor was used to study the degradation of filter paper cellulose and the activity of cellulolytic enzymes. The observed cellulolytic enzyme activities were lower than the enzyme levels necessary for the degradation of the amount of filter paper digested. Therefore the influence of the different steps of the procedure, used to prepare these enzyme preparations, on neutral detergent fibre degradation, fermentation product and reducing sugar formation was studied. Enzyme levels measured in enzyme preparations were about ten times lower than the levels calculated from the performance of the intact system.

INTRODUCTION

Carbohydrates of vegetable origin are fermented efficiently in the rumen. Bacteria, protozoa and phycomycetous fungi present in the rumen produce cellulolytic enzymes, which are capable of degrading highly ordered celluloses, such as cotton and filter paper cellulose (van Soest, 1973; Weimer et al, 1990). The digestion of cellulose involves an effective interaction of cellulolytic and non-cellulolytic organisms (Bryant, 1973). This is substantiated by the fact that even when cellulose is the only energy source in the diet, the cellulolytic bacteria represent only a quarter or less of the viable bacteria (Slyter et al, 1971).

Low cellulase activities are found in cell-free rumen fluid, which suggests that most of the cellulases produced by rumen microorganisms are either cell associated or tightly adsorbed to solid substrates (Halliwell, 1957; Akin, 1980; Williams & Strachan, 1984). Attachment of bacterial cells to plant surfaces is important in the degradation of fibre in the rumen. This attachment is mediated by the glycocalyx, a network of polysaccharides that extends from the bacterial surface (Lin et al, 1985). The cellulolytic enzymes are located in this glycocalyx (Lamed & Bayer, 1988). Protozoal enzymes are principally intracellular (Williams et al, 1986) although some release of enzyme activity does occur (Akin & Amos, 1979; Williams, 1979). Cellulolytic enzyme activities of phycomycetous fungi were detected both associated with the vegetative material and in cell-free culture supernatants (Williams & Orpin, 1987a; Wood et al, 1986). Their penetrative mode of growth, however, may ensure that the secreted enzymes act in the immediate vicinity of the fungus and minimizes the dispersion and the loss of these enzymes into the bulk of the rumen fluid (Williams & Orpin, 1987b).

In order to study the enzymatic activities against specific polysaccharides in crude rumen contents the polysaccharide-degrading enzymes have to be released from substrates and organisms. Several methods, as sonication, chilling, use of surfactants and substrate analogues, have been used to detach ruminal cellulolytic bacteria from the substrate (Minato & Suto, 1978; Akin, 1980; Leedle et al, 1987). Protozoa in rumen fluid can be disrupted by sonication and most of the cellulolytic activity produced by these organisms will be released into the medium (Coleman, 1985).

The present study was conducted to establish the effect of the procedure, used to extract cellulolytic enzymes from crude contents of an artificial rumen reactor, on the cellulolytic activity measured. Because considerable differences exist between cellulolytic activities in the artificial rumen, calculated on degradation efficiencies of pure cellulose, and activities measured in enzyme preparations from these rumen contents the effect of the individual steps in the procedure to extract cellulolytic enzymes on cellulose degradation, volatile fatty acid production and the formation of reducing sugars was tested.

MATERIALS AND METHODS

Treatment of the samples

An artificial rumen system was run as described in Chapter 2 and loaded with filter paper cellulose (15 $g.l^{-1}.d^{-1}$) and alfalfa hay (2 $g.l^{-1}.d^{-1}$). A homogeneous sample (900 ml) was taken sixteen hours after the addition of the substrate. This sample was cooled to 4°C to prevent denaturation of the enzymes due to heat generated during sonication. Part of the sample (300 ml) was used as such in the tests and is called homogeneous culture content (fraction A). The other part of the sample was sonicated in a Schoeller & Co TG 250 sonifier (Frankfurt a.M., Germany) in the presence of glass beads for 10 - 20 times 60 s with 45 s intervals and constant cooling on ice till no protozoa could be detected microscopically. Part (300 ml) of the sonicated sample was used as such (fraction B). The remaining part (300 ml) was centrifuged at 48,000 x g for 30 min at 4°C. The supernatant fraction was used as such (fraction C). The remaining residue was resuspended in 300 ml 50 mM Na_2HPO_4, pH 6.5, and indicated as residue resuspended in buffer (fraction D).

A schematic diagram of the treatment is given in Fig. 1.

Incubations

Digestion of the cellulose by fractions A, B, C and D was studied in batch incubations at 39°C. The effect of the sample treatment on neutral detergent fibre (NDF) degradation, on the production of volatile fatty acids (VFA), reducing sugars and protein formation and on pH was tested. The filter paper present in the samples was used as substrate but when supernatants were tested 2.5 g filter paper cellulose (FPC) per 300 ml was added.

NDF was determined in triplo at the start and the end of the experiment (after 24 h), VFA every hour during the first 6 - 8 hours of the incubation and after 24 h, protein after 0, 6 and 24 h, reducing sugars every 15 - 30 min during the first four hours of incubation and every hour after that. All analyses were performed as described in Chapter 2.

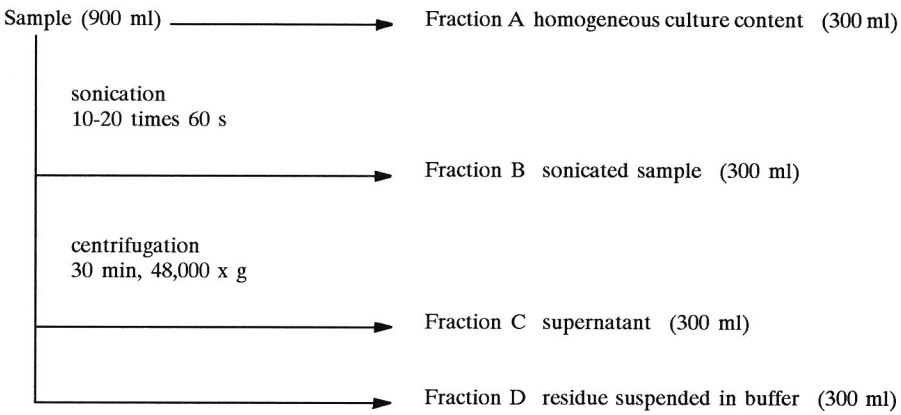


Fig. 1. Schematic diagram of sample treatment.

RESULTS

Degradation of filter paper cellulose in the artificial rumen reactor

The degradation of filter paper cellulose in the artificial rumen reactor was studied at a loading rate of 15 g FPC and 2 g alfalfa.$l^{-1}.d^{-1}$. The hydraulic retention time was kept at 12.5 ± 1.0 h and the solid retention time at 72 h. The results are given in Table 1.

Table 1. Performance of the artificial rumen reactor during steady state degradation of FPC (n=5)

Determination	
NDF degradation (%)	74 ± 7
VS degradation (%)	74 ± 8
VFA production (mmol.$l^{-1}.d^{-1}$)	91 ± 10
molar % acetic acid	64 ± 5
propionic acid	30 ± 4
butyric acid	5 ± 1
pH	6.5 ± 0.4
biogas production (l.$l^{-1}.d^{-1}$)	3.0 ± 0.9
methane production (l.$l^{-1}.d^{-1}$)	0.7 ± 0.2
protein (g.l^{-1})	0.84 ± 0.22

On basis of the NDF degradation measured (67 - 85%) the cellulolytic capacity of the reactor can be calculated. An amount of 10 - 13 g FPC.l^{-1}.d^{-1} is digested in the reactor or on protein basis 8 - 10 μg cellulose.mg protein^{-1}.min^{-1}. This is equivalent to the release of 51 - 64 nmol glucose.mg protein^{-1}.min^{-1}.

Determination of the total extractable cellulolytic activity

Sonication is an effective and generally used means to release cellulolytic activities from (artificial) rumen samples. The sonication procedure described in Chapter 2 was used to release the cellulolytic activities from a sample from an artificial rumen reactor loaded with FPC. The activities on avicel, carboxymethylcellulose and p-nitrophenyl-β-D-glycopyranoside of three consecutive extractions of this sample are given in Table 2.

Table 2. Enzymatic activities on carboxymethylcellulose (CMC), avicel and p-nitrophenyl-β-D-glycopyranoside (PNPG), released in three successive extraction steps.

Substrate	Number of extractions		
	I	II	III
CMC	154	77	68
Avicel	2	2	1
PNPG	19	7	5

Values are given in nmol reducing sugar or p-nitrophenol released.mg protein^{-1}.min^{-1} and are means of duplicate experiments

When samples were sonicated for 150 s (extraction I) 40 - 60 % of the total extractable activity on CMC, avicel and PNPG was released into the supernatant. When the pellet was resuspended in the original volume of fresh demineralised water and the procedure was repeated (extraction II) 20 - 40 % of the extractable activity was found in this supernatant. Repeating the sonication procedure a third time (extraction III) released another 15 - 25 %. This procedure is used to estimate the total extractable cellulolytic activity in artificial rumen samples. If the activity on avicel, a micro-crystalline cellulose, is taken as a standard for the degradation of filter paper cellulose 0.8 μg cellulose.mg protein^{-1}.min^{-1} is digested. Compared to the activity calculated from the performance of the intact artificial rumen system (8 - 10 μg cellulose.mg protein^{-1}.min^{-1}) the total extracted activity is approximately ten times lower.

Batch experiments

In order to study the effect of the procedure used to extract cellulolytic enzymes from artificial rumen samples on the activity measured the influence of the different steps of this procedure was determined (see Fig. 1). The effect of sonication and centrifugation on NDF-degradation (as a measure of cellulose degradation), VFA-formation, release of reducing sugars, pH and protein formation was studied in batch incubations. The results are presented in Table 3.

Table 3 NDF degradation, VFA-, protein- and reducing sugar formation and pH in batch incubations.

Sample	NDF degradation (%)	VFA formation ($mmol.l^{-1}$)	Reducing sugar formation between t=0 h and t=4 h ($nmol.ml^{-1}.min^{-1}$)
Fraction A	35 ± 10	53 ± 13[a]	0[b]
Fraction B	< 10	0	8 ± 3
Fraction C	< 10	0	5 ± 2
Fraction D	< 10	0	3 ± 1

	pH t=0 h	pH t=24 h	Protein ($g.l^{-1}$) t=0 h
Fraction A	6.6 ± 0.1	5.7 ± 0.2	0.84 ± 0.16
Fraction B	7.0 ± 0.2	7.2 ± 0.3	0.97 ± 0.18
Fraction C	6.6 ± 0.2	6.7 ± 0.2	0.50 ± 0.17
Fraction D	7.0 ± 0.2	7.7 ± 0.2	0.48 ± 0.27

Values are expressed as means ± SD. Homogeneous culture contents (Fraction A) n = 14, sonicated sample (Fraction B) n = 3, residue suspended in buffer (Fraction C) n = 4, supernatant (Fraction D) n = 4

[a] Molar ratio of VFA produced between t=0 h and t=24 h : A:P:B:IV:V=55:38:6:1:1

[b] Velocity of reducing sugar formation during the first four hours of incubation

When rumen contents were disrupted by sonication no NDF degradation (taken as a measure of cellulose solubilization) could be measured within 24 hours. On basis of the amount of reducing sugars liberated during the first four hours of incubation in a sonicated sample a NDF degradation of 15 % was to be expected. However, after approximately 4 - 6 hours incubation the rate of reducing sugar formation declined. During the first six hours of incubation some VFA formation took place in the

sonicated sample but these VFA were degraded in the period between 6 and 24 hours. In the homogeneous culture contents no reducing sugars accumulated. In all sonicated and resuspended preparations and the supernatant fraction reducing sugars were formed in low concentrations (up to maximal 4 mM) during the first 4 - 6 hours of incubation.

DISCUSSION

Total ruminal digestion is often underestimated by the measurement of enzymatic digestion. Pure cultures of rumen microorganisms are usually not able to degrade plant material as quickly as either whole rumen contents or cocultures of suitable microorganisms (Chesson et al, 1986; Owens, 1987; Kopecny & Williams, 1988). Cellulosic substrates are degraded efficiently by a mixed ruminal flora, irrespective of the crystallinity of these substrates. Synergistic activities of the different cellulolytic species which make up the ruminal microflora may be responsible for this efficient degradation (Weimer et al, 1990).

Besides the effects of microorganisms the synergistic action of the different enzymes of the cellulase complex, which by themselves are essentially inactive towards microcrystalline cellulose, is essential for the degradation of this substrate (Eveleigh, 1987). The adhesion of ruminal bacteria to the substrate and the presence of cellulolytic enzymes on the bacterial cell surface in an optimal configuration towards the substrate are essential in the digestion of complex substrates such as plant cell walls or cellulose (Morris & Cole, 1987).

Ruminococcus-species usually form an extracellular high molecular weight complex in which the cellulolytic enzymes of these bacteria can degrade microcrystalline cellulose. When this complex is not formed the individual cellulases are free to diffuse from the cell and are inactive against microcrystalline cellulose in this uncomplexed state (Robson & Chambliss, 1989). With *Bacteroides succinogenes* the presence of intact cells also seems to be required to achieve a significant degradation of highly ordered cellulosic substrates. Their cellulases are probably associated with the cell envelope (Robson & Chambliss, 1989).

It has been shown that only very small proportions, often less than 15 % of the total cellulase, hemicellulase and glycosidase activities are found in the cell-free culture fluid of rumen contents (Williams & Strachan, 1984). To estimate the activity of (hemi) cellulases and glycosidases on specific substrates (e.g. carboxymethylcellulose, xylan, avicel) these enzymes have to be released from organisms and substrates.

The rate-limiting step in the fermentation of cellulose in the rumen as well as in the artificial rumen reactor is the conversion of cellulose to soluble substrates that are fermented as rapidly as they are formed. In the artificial rumen reactor the concentration of reducing sugars is low and amounts to 0.2 ± 0.1 mM. Since the level of reducing sugars is constant cellulolytic activities have to be calculated from degradation efficiencies.

Samples from an artificial rumen system fed filter paper cellulose were used to estimate the effect of separate steps in the sonication procedure, used to extract

cellulolytic enzymes, on cellulose (NDF) degradation, volatile fatty acid formation and the release of reducing sugars.

Based on degradation efficiencies (65 - 80 %) measured in the artificial rumen system cellulolytic enzyme activities should be high. The expected enzyme levels amount to 50 - 61 nmol reducing sugar set free per mg protein per min. In contrast to this, the measured activities, based on the formation of reducing sugars from FPC by sonicated preparations amount to only 8 nmol per mg protein per min. The combined cellulolytic activity on avicel, a commercial cellulose and like FPC not highly crystalline (Eveleigh, 1987), determined in supernatants of three successive sonication steps amounts to only 5 nmol per mg protein per min.

Lubberding et al (1987) have shown that sonication is an effective means to release cellulolytic activities from artificial rumen samples. Treatment with detergents, whether or not in combination with sonication gave no significant increase of the total activity released. Extending the sonication time above 150 s had only a minor effect on the amount of extracted enzymes. This is in accordance with results from Silva et al (1987), who concluded that sonication was at least as efficient as other, chemical methods of extraction of particle-bound cellulolytic enzymes from rumen microorganisms.

However, cellulolytic activities measured in sonicated samples of the artificial rumen reactor are about ten times lower than activities calculated on degradation efficiencies. The disruption of the cellulolytic complexes on the bacterial cell walls and the solubilization of the enzymes, present in special configurations in these complexes, prevent the synergistic action of these enzymes. Moreover, there is no evidence that all enzyme activity has been released from the microorganisms or plant debris by the sonication treatment, as some activity may still be bound in an inactive form to particulate matter (Coleman, 1985), or inactivated by the treatments applied.

In conclusion, synergistic effects between cellulolytic enzymes, usually embedded in cellulolytic complexes, are important in (artificial) rumen samples. These complexes are disrupted by sonication resulting in a decreased enzymatic activity. Therefore enzyme measurements on sonicated samples will give an underestimation of the cellulolytic potential of the system.

POLYSACCHARIDE-DEGRADING ENZYME ACTIVITIES IN AN ARTIFICIAL RUMEN SYSTEM LOADED WITH CELLULOSE AND PRODUCTS FROM OILSEED INDUSTRY

SUMMARY

An artificial rumen system was loaded with filter paper cellulose and low protein soybean and rapeseed meal. Degradation of these substrates and the induction of polysaccharide-degrading enzyme activities were tested. Although the chemical composition of these substrates showed marked differences, no differences in specific enzyme activities were observed.

INTRODUCTION

The rumen represents a natural cellulolytic ecosystem, in which microorganisms degrade plant polysaccharides with high efficiency. The degradation of these polysaccharides is achieved by the interaction of polysaccharide depolymerase and glycoside hydrolase enzymes produced by bacteria, protozoa and anaerobic fungi (Hobson, 1988).

The effect of diet composition and intake on rumen fermentation is largely determined by the rate of fermentation of the individual dietary components and the end products produced by the microflora. Soluble sugars are the most rapidly fermented components of diets, while protein and starch are degraded more slowly; the rate of their degradation varies depending on the source of protein or starch, the processing of the feedstuff and changes in the rumen environment. In contrast, fibre degradation is the slowest process and often rate-limiting (Berger, 1988).

The composition of the substrates in the rumen environment may regulate levels of enzymes, involved in polysaccharide breakdown, produced by individual bacterial strains (Russell & Baldwin, 1978; Williams & Withers, 1982). Changes in numbers and kinds of microorganisms, however, are probably the major effect of dietary components in the rumen (Leatherwood, 1973). Drastic changes in the bacterial population of sheep ruminal fluids in the presence of different feeds during incubation *in vitro* were shown by Gonzalez-Lopez et al (1990); *in vivo* such changes were described by Varel & Dehority (1989).

Recently, an artificial rumen reactor was developed (Gijzen et al, 1986). This reactor fed a grass / grain mixture comparable to animal feed resembles well the *in vivo* rumen system, with respect to microorganisms, fermentation products, degradation efficiency and even the typical odour. For this reason the artificial rumen reactor is suitable to study the influence of dietary components on the levels of polysaccharide degrading enzymes under standardized conditions.

In this study the artificial rumen reactor was used to investigate the influence of feeding filter paper cellulose, soybean meal and rapeseed meal on enzyme activities. The potential of this system to degrade complex plant materials was discussed in Chapter 3. The question to be solved was, if single test substrates would induce a special population of microorganisms producing "tailor-made" enzymes for polysaccharide degradation.

MATERIALS AND METHODS

Enzyme preparation

Four hours after the addition of substrate a homogeneous sample was taken from the reactor and cooled to 4°C. Microbial cells were disrupted by ultrasonic disintegration (Branson B12, Danburg Connecticut) at 4°C in the presence of glass beads (Ø 4 mm). After ten times 30 s of sonication with 30 s intervals, the mixture was centrifuged at 48,000 x g for 30 min at 4°C. The supernatant was used as the enzyme preparation.

Enzyme assay procedures

The polysaccharide substrates used to test enzyme activities were carboxymethylcellulose, oat spelt xylan, locust bean gum, arabinogalactan, citrus pectin (Sigma), avicel (Serva), filter paper cellulose (Whatman, grade 91) and the neutral detergent fibre (NDF) fractions of defatted soybean meal and defatted rapeseed meal (prepared according to Goering & van Soest,1970). Xylan was washed three times in demineralised water and lyophilised before use.

Degradation of polysaccharides was determined by measuring the amount of reducing sugars released from the various substrates (0.5 % w/v in 0.1 M Na-acetate buffer pH 5.5). The incubation mixture contained 40.0 ml substrate-buffer solution and 10.0 ml enzyme preparation; the pH of the incubation mixture was adjusted to 5.5 when necessary. Incubations were performed at 39°C while shaking the mixtures. Duplicate samples were taken at 5 to 15 min intervals during a period of 40 min to 4 h depending on the activity. During this period no degradation of released sugars occurred. Reducing sugars were quantified by the method of Nelson and Somogyi (Somogyi, 1952) with the appropriate monosaccharide standards.

RESULTS

Digestion of the substrates in the artificial rumen reactor

Degradation efficiency was studied at loading rates of 15.7 (filter paper cellulose), 31.4 (low protein soybean meal) and 39.8 (low protein rapeseed meal) g volatile solids.l^{-1}.d^{-1} with a solid retention time of 72 h and a hydraulic retention time of 12.0 ± 1.0 h. With these loading rates the NDF load of the three systems is about equal : 15.0 ± 0.5 g NDF.l^{-1}.d^{-1}. Degradation on the basis of NDF and volatile solids (VS), pH and biogas production is shown in Table 1, volatile fatty acid (VFA) production in Table 2.

The NDF- and VS-degradation is high in all cases (above 60 %). When loading with FPC only acetic, propionic and butyric acid were produced, whereas with LPSM and LPRM isobutyric, isovaleric and valeric acid were produced as well.

Table 1. Steady state degradation of the substrates, pH and biogas production in the artificial rumen reactor

Substrate	pH	Degradation (%)		Biogas-production	
		NDF	VS	$l.l^{-1}.d^{-1}$	$l.g^{-1}$ VS digested
FPC	6.7 ± 0.1	69	65	2.4 ± 0.5	0.23 ± 0.05
LPSM	6.8 ± 0.1	94	81	5.2 ± 0.5	0.19 ± 0.02
LPRM	6.7 ± 0.1	62	64	4.1 ± 0.5	0.15 ± 0.02

Abbreviations : FPC, filter paper cellulose; LPSM, low protein soybean meal; LPRM, low protein rapeseed meal

Table 2. Production of volatile fatty acids during steady state degradation of different substrates

Substrate	VFA-production		molar %						
	$mmol.l^{-1}.d^{-1}$	mmol.g VS $digested^{-1}$	A	P	IB	B	IV	V	MV
FPC	89 ± 15	8.5 ± 1.4	60	33	1	6	0	0	0
LPSM	91 ± 16	7.0 ± 0.6	54	19	3	11	7	5	1
LPRM	160 ± 12	5.8 ± 0.4	55	24	3	9	5	4	1

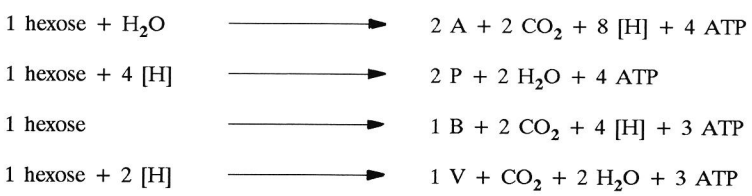
Abbreviations : A, acetic acid; P, propionic acid; IB, isobutyric acid; B, butyric acid; IV, isovaleric acid; V, valeric acid; MV, methylvaleric acid

Estimation of biomass

To compare the specific activities of the enzyme preparations from the LPSM- and LPRM-cultures the protein contents of these cultures had to be calculated since direct measurements were not possible due to the high protein content, largely originating from the substrate. Calculations were performed using the protein / fermentation product ratio obtained from the reactor fed FPC. In this reactor the measured amount

of protein will be accounted for by the microbial biomass only, since the substrate contains no protein.

The protein content can also be calculated from the amount of ATP produced in the conversion of carbohydrate to the fermentation products (volatile fatty acids and methane) (Hobson & Wallace, 1982b). In the formation of acetic (A), propionic (P), (iso)butyric ((I)B), (iso)valeric ((I)V) acid and methane (CH_4) 2, 2, 3, 3, 1 mol ATP are formed, respectively (Hungate, 1988).



Methane production was calculated from the reducing equivalents generated in the formation of acetic and butyric acid and consumed in the formation of propionic and valeric acid. Assuming an Y_{ATP} of 10.5 g dry weight microbial biomass/mol (Bauchop & Elsden, 1960) and a biomass protein content of 45 % (Smith, 1975) the microbial protein content of the three different cultures was calculated.

The results of the biomass calculations are summarized in Table 3. The measured protein content for the reactor fed FPC does not differ significantly from the calculated one (from ATP formation), indicating that assumptions made were adequate.

Table 3. Calculation of biomass-protein in the different reactors

Substrate	Total VFA-production ($mmol.l^{-1}.d^{-1}$)	Protein ($g.l^{-1}$) (from VFA-protein ratio)	Protein ($g.l^{-1}$) (from ATP-formation)
FPC	86 ± 24[a]	0.84 ± 0.22[b]	0.94 ± 0.28
LPSM	205 ± 16	1.93 ± 0.70	2.35 ± 0.22
LPRM	163 ± 26	1.54 ± 0.60	1.90 ± 0.30

Loading rate 15 g $NDF.l^{-1}.d^{-1}$ for all substrates
[a] Values are expressed as means ± SD. For FPC n = 46, LPSM n = 24, LPRM n = 21
[b] Measured by protein determination

Enzyme activities

The specific activities of polysaccharide depolymerases on carboxymethylcellulose, filter paper cellulose, avicel, glycogen, oat spelt xylan, pectin, locust bean gum,

arabinogalactan and NDF-fractions of LPSM and LPRM were determined in enzyme preparations from the cultures loaded with the different substrates.

The initial velocity of the formation of reducing sugars and the protein content measured or calculated (Table 3) was used to estimate the specific activities of the polysaccharide depolymerases of the different enzyme preparations (Table 4).

Table 4. Specific activities of the polysaccharide depolymerases in the different enzyme preparations

Substrate	Enzyme preparation		
	FPC	LPSM	LPRM
Carboxymethylcellulose	95[a]	28	105
Filter paper			
ball milled	1	2	4
shredded	2	2	5
Avicel	4	2	5
Glycogen	11	15	29
Xylan	120	62	63
Pectin	24	28	40
Locust bean gum	32	19	67
Arabinogalactan	2	4	9
NDF-fraction LPSM	2	3	6
NDF-fraction LPRM	2	3	11

[a] Values in nmol reducing sugar released.mg protein^{-1}.min^{-1} and expressed as initial velocities based on at least eight measurements in duplo

The results in Table 4 show that, although the reactors were loaded with different substrates, the activity profile of the polysaccharide-degrading enzymes is similar. High activities were found with carboxymethylcellulose, xylan, pectin, locust bean gum and glycogen as substrates.

DISCUSSION.

Ruminal digestion of plant materials involves numerous complex processes carried out by a variety of microbial species and enzymes. Overall digestion of the plant material is affected by a number of intrinsic plant factors (e.g. physical form, fibre composition) and these factors together with pretreatment will determine the residence time in the rumen (Hespell, 1988).

Compared to the *in vivo* system *in vitro* conditions are more standardized and reproducible (Gijzen et al, 1986) and allow the use of specific hydraulic and solid retention times to study the effect of certain dietary components on degradation and enzymatic activities in the rumen. Digestibility studies in an artificial rumen system show that fibre and VS degradation are very efficient although the substrates differ markedly in composition. According to Gijzen et al (1987) LPSM and LPRM can be typed as materials rich in cell solubles (CS-rich; more than 40 % of the VS consist of cell solubles) and FPC as a cellulosic substrate (more than 50 % of the VS consist of cellulose and less than 40 % of cell solubles). The VFA pattern should therefore differ between these groups of substrates. Acetic acid is in both cases the major product. On CS-rich substrates relatively high butyric, isovaleric and valeric acid concentrations were found, while cellulosic substrates were characterized by higher propionic acid concentrations and the absence of C_5 - C_7 acids. The production of isovaleric and valeric acid may originate from the remaining protein present in LPSM and LPRM (Hungate, 1966). The values obtained with FPC as a substrate resemble well those reported by Gijzen et al (1987).

Studies with ruminal bacterial strains have indicated that the amount of polysaccharide degrading enzyme activities can be influenced by the growth substrate (Hespell et al, 1987; Williams & Withers, 1982). Substrate preferences and sequential utilization have been found in several bacterial strains from the rumen and are important in the competition between these bacteria (Russell & Baldwin, 1978). Extensive interaction between micro-organisms is involved in the degradation of polysaccharides in the rumen. Production of polysaccharide-degrading enzyme activities is limited to a few species, but competition for the products of the hydrolysis of these polysaccharides sustains the growth of many species, incapable of the direct use of these substrates (Wolin & Miller, 1988).

This investigation shows that the specific activities measured in the artificial rumen reactor fed with the different substrates appear to be similar. Although the reactors were loaded with filter paper cellulose, a model substrate consisting of 100 % cellulose, and crude plant derived material, surprisingly no differences in polysaccharide depolymerase enzyme activities were observed. In reactors fed with FPC, high xylan and locust bean gum (a galactomannan polymer) depolymerising activities were measured, although no induction of these enzymes is expected from these substrates.

Gattinger et al (1990) used rapeseed meal as a substrate for xylanase production by *Trichoderma reesei* (an aerobic fungus) and showed that better xylanase yields were obtained than from substrates such as Solka-floc, oat spelt xylan or glucose. In the artificial rumen reactor relatively high enzyme-activities on locust bean gum and pectin were obtained when LPRM is used as a substrate. Specific activities on NDF-fractions from rapeseed- and soybean meal were somewhat higher in this reactor compared to the reactor on FPC, possibly indicating a slight shift in microorganisms or enzymes present depending on the diet.

The influence of the diet on the composition of the microbial flora in the rumen might be limited since high numbers of prominent cellulolytic bacteria, such as *Ruminococcus albus* and *Bacteroides succinogenes*, were even found in ruminants fed on diets that contain much starch and little cellulose (Latham et al, 1971). Since these

microorganisms do not use maltose and many strains do not use glucose, even a small amount of cellulose in the diet may prevent a severe reduction in the numbers of the cellulolytic species. Williams et al (1989) showed that the activities of polysaccharide degrading enzymes in the various populations separated from rumen contents changed during the postprandial period. Total enzyme activity four hours after feeding is approximately 10 fold lower on carboxymethylcellulose, 5 fold lower on pectin and approximately the same on xylan compared to the various enzyme-preparations from the artificial rumen reactors. Specific activities measured by Williams & Strachan (1984) in crude digesta are 10 to 15 fold lower than our values.

The sonication procedure used to dissociate the polysaccharide-degrading enzymes from microorganisms and substrates gives an underestimation of the activities in the system (Chapter 4). This technique, used by Williams & Strachan (1984), Williams et al (1989) and ourselves may influence the measured activities since it does not release all enzymes and probably disrupts the complexes in which the enzymes are embedded. However, the same technique is used for all experiments and so comparison of the results is possible.

In conclusion, this paper shows that specific enzymatic activities in the *in vitro* rumen ecosystem may vary depending on the feeding substrate but changes in the activity pattern of the polysaccharide degrading enzymes induced by the diet are minimal.

IMPROVEMENT OF SOYBEAN AND RAPESEED OIL YIELD BY PRETREATMENT WITH FRACTIONS FROM AN ARTIFICIAL RUMEN SYSTEM, ENZYMES FROM AN ANAEROBIC FUNGUS AND A COMMERCIAL ENZYME PREPARATION

SUMMARY

The potential of fractions from artificial rumen reactors loaded with low protein soybean meal, low protein rapeseed meal and filter paper cellulose, of a commercial preparation (Viscozyme^{TM}L, NOVO) and of an enzyme preparation from an anaerobic fungus (*Piromyces* sp.) to improve oil yield from soybean flakes and rapeseed was tested. The total oil yield obtained in the treatments was comparable. The specific oil yield, however, was considerably higher when *Piromyces* sp. enzyme or the commercial preparation were used, compared to the artificial rumen fractions. Loading the artificial rumen reactor with oilseed fractions enriched in cell walls had little effect on the improvement of the oil yield.

INTRODUCTION

The bulk of fats and oils, whether for human consumption or industrial usage, is derived from plants (Pryde & Doty, 1981). Soybean, palm, sunflower and rapeseed are the major sources of plant oil (Fenwick, 1984). Soybean oil is the major edible oil used in the world (Erickson, 1983). Mature soybeans contain 18 to 20 % (w/w) oil (Pryde & Doty, 1981) with a relatively high content of linolenic acid (Rattray, 1984). To obtain crude soybean oil the beans are cleaned, cracked, dehulled and conditioned. Moisture is adjusted to 10 - 11 % and temperature to 65 - 70°C during conditioning. Beans are flaked to 0.010 - 0.012 inch thickness, to ensure efficient extraction. Flakes are then extracted with hexane in a percolation extractor to an uniform residual oil content (0.5 to 1.0 % (w/w)) (Becker, 1971; Garcia Serrato, 1981).

In contrast to soybeans, which are derived from one species (*Glycine max* (L)) rapeseed may come from several species, generally belonging to the genus *Brassica* (Bengtsson et al, 1972). Rapeseed from Europe usually belongs to the species *Brassica napus* (winter and summer types of rape), rape from Canada to the species *Brassica campestris* (turnip rape). These types are morphologically similar but physiologically very different as for instance the winter type does not form seeds if the crop has not been exposed to subzero temperatures for a certain period of time (Bengtsson et al, 1972). Rapeseed has a higher oil content than soybeans : 40 - 45 % (w/w) (Pryde & Doty, 1981). Two major types of rapeseed oil are produced : LEAR (low erucic acid rapeseed) for edible use and HEAR (high erucic acid rapeseed) for industrial applications (Rattray, 1984). To obtain crude oil from rapeseed the material is usually pressed followed by solvent extraction (Anjou, 1972). The seed is cleaned and then dried to prevent hydrolysis of the glucosinolates, by myrosinase present in the seed, during crushing. Inactivation of myrosinase by cooking is essential to obtain oil and meal of good quality and free of glucosinolate hydrolysis products. Cooking also coagulates proteins, destructs bacteria, increases fluidity of the oil and reduces affinity of the oil for solid surfaces (Anjou, 1972). After pressing in a mechanical screw press the cake (oil content 12 - 20 %) is cooled, moistened and milled to a meal that can be solvent extracted with hexane (Anjou, 1972).

The processes preceding pressing and / or solvent extraction are designed to mechanically disintegrate the cell walls and release the oil droplets more efficiently. The efficiency of the oil extraction processes can also be increased by treatment of the crushed rapeseed or soybeans with mixed enzyme preparations from *Aspergillus niger* and *Bacillus subtilis* prior to solvent extraction (Fullbrook, 1983). The use of special enzymes in oil extraction processes gives a 2 - 6 % increase in the total amount soxhlet extractable oil (Godfrey, 1983; Bhatnagar & Johari, 1987), with additional benefit in lower oil content in the final seed cake feedstuffs (Godfrey, 1983). Aqueous processing of rapeseed and soybean flakes will also remove phytic acid and other toxins from the remaining protein meal, making it a more useful byproduct (Fullbrook, 1983).

This study was performed to investigate the use of fractions of an artificial rumen system to enhance solvent extraction of soybean flakes and ground rapeseed. For comparison the cellulolytic enzymes produced by an anaerobic fungus and a commercial enzyme preparation (Viscozyme^{TM}L, NOVO) were also tested. The artificial rumen systems were loaded with soybean and rapeseed substrates and pure cellulose to test the induction of cell wall degrading enzymes, specially suited to degrade soybean and rapeseed cell walls.

MATERIALS AND METHODS

Substrates

Soybean flakes were used as such, rapeseed was frozen in liquid nitrogen, milled in a coffee grinder and cooked for 20 minutes (33 % w/w in water). Oil contents of the substrates were determined by soxhlet extraction with hexane for 6 hours at about 60°C. After evaporation of the hexane the oil was dried to a constant weight at 105°C.

Soybean flakes and ground cooked rapeseed contain 20.7 ± 3.3 % and 51.3 ± 1.2 % oil on basis of the total solids, respectively.

Enzymes and fractions used for the enhancement of oil disclosure

Incubations were performed with homogeneous culture content from artificial rumen reactors loaded with filter paper cellulose (FPC), low protein soybean meal (LPSM) and low protein rapeseed meal (LPRM). The loading rate of these cultures was about equal on basis of crude fibre (Chapter 5). Degradation efficiencies of the substrates at these loading rates are given in Chapter 5. A sample from the homogeneous culture content (50 ml) was concentrated by centrifugation (30 min, 48,000 x g) followed by resuspension of the residue in 25 ml of incubation buffer.

The commercial enzyme preparation used, Viscozyme^{TM}L, was a gift from NOVO Industri A/S (Bagsvaerd, Denmark). This polysaccharidase preparation is especially suited for plant cell wall degradation and is produced by a selected strain from the

Aspergillus group. The enzyme preparation was used at a concentration of 4 % w/w (140 mg per 3.5 g substrate) as advised by NOVO.

The anaerobic fungus *Piromyces* sp. strain IEL (Teunissen et al, 1991a) was kindly supplied by M. Teunissen. The culture filtrate of this strain, grown on filter paper cellulose, was used as an enzyme preparation without pretreatment. Incubations were performed with 12.5 ml culture filtrate mixed with 12.5 ml buffer of double strength.

Enzymatic disclosure of soybean and rapeseed oil

To measure the enzymatic disclosure of oil 3.5 g soybean flakes or ground, cooked rapeseed were incubated in a total volume of 25 ml present in closed 100 ml serum bottles. Several types of buffer were used for the incubations : 0.5 M sodium phosphate, pH 6.8 and 0.5 M sodium bicarbonate, pH ± 7.0 under 100 % CO_2 for incubations with artificial rumen samples; 0.1 M citric acid/ 0.2 M phosphate at pH 4.5 for incubations with Viscozyme^{TM}L and at pH 6.0 for *Piromyces* sp. When 0.5 M sodium phosphate or sodium bicarbonate was used in incubations with artificial rumen contents the pH of the incubation mixture did not fall below 5.8 during the incubation time. In incubations with Viscozyme^{TM}L and *Piromyces* sp. enzyme the pH remained close to the set value during incubation.

Incubations were performed at 39°C (artificial rumen samples and *Piromyces* sp.) or 45°C (Viscozyme^{TM}L) for 0, 4 and 7 hours with shaking at 150 rpm. All incubations were carried out in triplicate. At the end of the reaction time 30 ml 96 % ethanol and 20 ml n-hexane were added and mixed thoroughly after each addition. After centrifugation (10 min; 12,000 x g) the hexane layer, containing the released oil, was transferred to a weighed erlenmeyer. The hexane was evaporated by pressured air; the remaining oil was dried for 1 hour at 105°C and weighed.

Routinely oil from incubation mixtures with rapeseed or soybean flakes was extracted with an ethanol / hexane mixture in this ratio (1.5). For very firm gels a ratio of 2.5 was used (50 ml ethanol and 20 ml hexane).

Determination of (hemi)cellulolytic activity and protein content of the enzyme fractions tested

The (hemi)cellulolytic enzymes in artificial rumen fractions were released in three successive sonication steps (see Chapter 2). The culture filtrate of *Piromyces* sp. and Viscozyme^{TM}L were cell free enzyme solutions. The protein contents of the *Piromyces* sp. culture filtrate, Viscozyme^{TM}L and the FPC fraction (after extraction) were measured, the protein content of the LPSM and LPRM fractions was calculated using the protein / fermentation product ratio obtained from the reactor fed FPC, as described in Chapter 5.

RESULTS

Effect of treated and untreated artificial rumen samples on oil disclosure

As is shown in Chapter 4 enzyme preparations from the artificial rumen system, prepared by sonication, were considerably less active than intact samples. The difference between treatment with an enzyme preparation (the supernatant of a homogeneous reactor sample, sonicated 10 times 30 s and centrifuged 30 min at 48,000 x g) and an intact sample from the artificial rumen reactor loaded with LPRM (both 50 ml) is shown in Table 1.

Table 1. Comparison of the oil yield from cooked rapeseed after pretreatment with homogeneous culture content and an enzyme preparation from an artificial rumen system loaded with LPRM.

	Oil Yield[a]	
Incubation time (h)	Homogeneous culture content	Enzyme preparation
0	18.7 ± 0.8[a]	14.0 ± 1.4
4	27.3 ± 1.4	17.8 ± 0.6
7	30.2 ± 1.9	20.5 ± 2.8

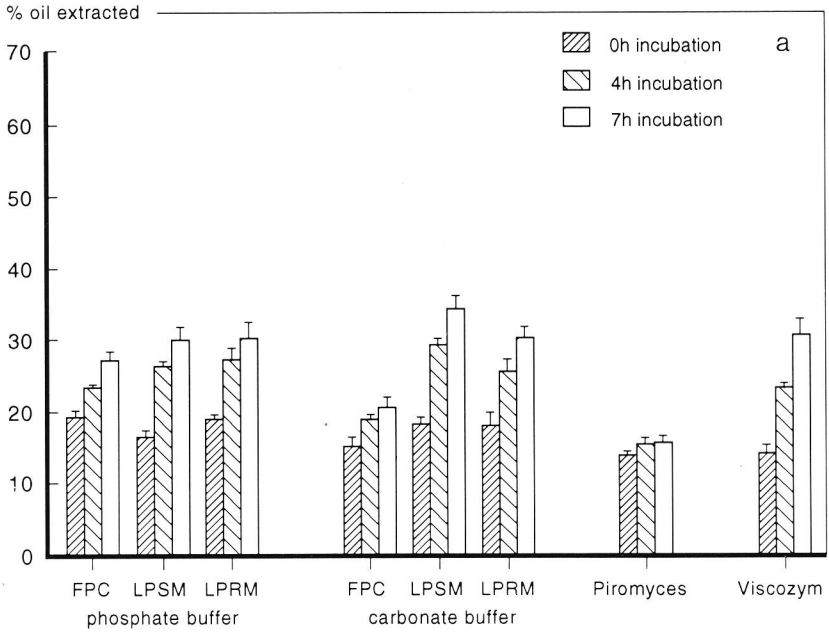
Incubations were performed in 0.5 M sodium phosphate buffer, pH 6.8
[a] Values are expressed as percents of the total amount soxhlet extractable oil

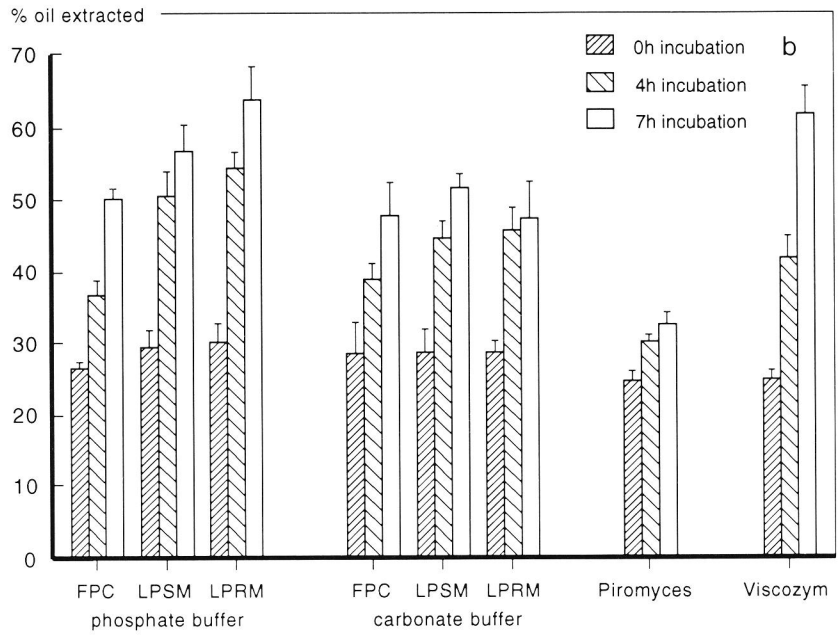
Since homogeneous culture content gives a higher oil yield and a better estimation of the potential of the system (see Chapter 4), all following experiments were done with these fractions.

Comparison of the improvement of soybean and rapeseed oil yield by enzyme preparations

The improvement of the oil yield from soybean flakes and cooked rapeseed by incubation with intact samples from artificial rumen systems, loaded with FPC, LPSM and LPRM, enzymes from *Piromyces* sp. and Viscozyme^{TM}L was tested. The results are given in Fig. 1.

Fig. 1. (Facing page) Oil yield after incubation with different enzyme preparations (as % of the total amount soxhlet extractable oil).
a : rapeseed, b: soybean flakes


% oil extracted
70
60
50
40
30
20
10
0
0h incubation
4h incubation
7h incubation
a
FPC
LPSM
LPRM
phosphate buffer
FPC
LPSM
LPRM
carbonate buffer
Piromyces
Viscozym


% oil extracted
70
60
50
40
30
20
10
0
0h incubation
4h incubation
7h incubation
b
FPC
LPSM
LPRM
phosphate buffer
FPC
LPSM
LPRM
carbonate buffer
Piromyces
Viscozym

Treatment of cooked rapeseed and soybean flakes with different enzyme preparations resulted in an increase in the total amount of extractable oil. This increase was more distinct in soybean flakes than in rapeseed.

Enhancement of oil disclosure from rapeseed and soybean flakes by artificial rumen preparations was comparable to that with Viscozyme^{TM}L. With the *Piromyces* sp. enzyme a relatively low oil disclosure from rapeseed was measured. Oil yield from soybean flakes by *Piromyces* sp. enzyme was considerably higher. However, Viscozyme^{TM}L was used in the concentration advised by NOVO (4 % w/w, 120 μl.25 ml^{-1}) whereas 50 ml artificial rumen fraction (concentrated to 25 ml) was used. For incubations with *Piromyces* sp. enzyme 12.5 ml was used in a volume of 25 ml. To compare the different fractions and enzyme preparations the specific oil disclosing activity was calculated (Table 2).

Table 2. Specific oil disclosure of the different preparations.

	oil yield	
culture fraction / enzyme	soybean flakes	cooked rapeseed
FPC	0.065[a]	0.025
LPSM	0.055	0.025
LPRM	0.081	0.029
Viscozyme^{TM}L	1.736	0.924
Piromyces sp.	0.830	0.262

Incubations in 0.5 M phosphate buffer; figures are corrected for oil yield with the enzyme preparation tested at t=0.

[a] Values are expressed as percents oil released per mg protein added per hour during the first four hours of incubation.

Specific oil disclosing activities were much higher with Viscozyme^{TM}L and *Piromyces* sp. enzyme than with the artificial rumen samples.

Cell wall degrading activities in the different enzyme preparations tested

For further comparison of the enzyme preparations tested, the (hemi)cellulolytic activity and the protein content of the various fractions were measured.

The polysaccharide degrading activities in a 200 times diluted Viscozyme^{TM}L preparation are comparable to those in artificial rumen samples. The activities in *Piromyces* sp. samples, however, are 2 - 3 times higher compared to artificial rumen

samples. Combined with the lower protein content of Viscozyme^{TM}L and *Piromyces* sp. culture filtrate the specific enzyme activities in these preparations are considerably higher than those in preparations from artificial rumen samples.

Table 3. Enzymatic activities and protein content of the different fractions used to improve oil-extraction

culture fraction / enzyme	CMC-ase	Xylanase	Avicelase	β-glucosidase	Protein
FPC[a]	244 ± 21[d]	264 ± 35	67 ± 1	25 ± 2	0.81 ± 0.18[e]
LPSM	268 ± 84	269 ± 64	12 ± 4	99 ± 14	1.93 ± 0.70
LPRM	233 ± 67	150 ± 13	15 ± 3	110 ± 4	1.50 ± 0.59
Viscozyme^{TM}L[b]	659 ± 118	83 ± 6	13 ± 1	89 ± 20	0.10 ± 0.02
Piromyces sp.[c]	432 ± 97	485 ± 85	37 ± 4	63 ± 3	0.13 ± 0.01

[a] enzymatic activities of artificial rumen samples are expressed as the sum of the activities of preparations from three successive sonication steps
[b] enzymatic activities and protein content in a 200 times diluted preparation; this dilution is comparable to the concentration used in oil disclosure experiments
[c] enzyme activities in the culture filtrate
[d] all enzyme activities are expressed in nmol product released.ml^{-1}.min^{-1}
[e] values expressed in mg.ml^{-1}

Direct comparison of the oil disclosure by the different enzyme preparations and the enzymatic activities of these preparations is difficult. The cellulolytic activity of the rumen fractions is underestimated when samples are sonicated to liberate cellulolytic enzymes (see Chapter 4), whereas the Viscozyme^{TM}L preparation and the *Piromyces* sp. sample enzymes are cell free enzyme solutions.

DISCUSSION

The use of cell wall degrading enzyme preparations to enhance oil extraction from oilseeds such as soybeans and rapeseed may lower energy requirements and solvent usage in oilseed processing (Fullbrook, 1983; Sosulski, 1986). An artificial rumen system loaded with oilseed meals was shown to produce all kinds of cell wall degrading activities (Chapter 5). Fractions of this system might therefore be suited to improve oil extraction.

The formation of protein gels when hexane was added to the reaction mixture for extraction hindered the measurement of oil disclosure by artificial rumen fractions. Without the addition of ethanol no oil disclosure was measured from soybeans. Soy proteins can form highly ordered gel structures (Hermansson, 1986; Shimada &

Matsushita, 1980; Shimada & Cheftel, 1988). Only a weak gel was formed when prior to the addition of hexane ethanol was added. This gel disappeared after centrifugation and oil disclosure could be measured. Addition of ethanol to the incubation mixture probably results in denaturation and precipitation of a part of the protein released during incubation with artificial rumen fractions.

The cellulolytic potential of enzyme preparations from the artificial rumen system was significantly lower than that of intact artificial rumen contents (Chapter 4). In agreement with this, the improvement of oil extraction by an enzyme preparation from an artificial rumen culture loaded with LPRM was about 50 % lower than the enhancement by intact culture contents of the same system.

A rather strong buffer (0.5 M) was needed to keep the pH of the incubation mixture between the physiological boundaries of the rumen (5.5 - 7.2). A phosphate and a carbonate buffer were tested. Although high phosphate concentrations are known to inhibit cellulolytic enzyme activities (Stewart, 1977), no negative effects of the high phosphate concentration were seen when compared to the carbonate buffer (Fig. 1). Francis et al (1978) reported that high phosphate concentrations (0.4 - 0.6 M) do not inhibit rumen cellulase activities.

The enhancement of oil extraction by enzymatic pretreatment is considerably lower in cooked rapeseed compared to soybean flakes. A higher oil yield from soybeans than from rapeseed after enzymatic treatment is also reported by Fullbrook (1983).

Comparison of the specific oil disclosing activity of the different preparations is difficult. For the artificial rumen fractions total biomass protein was measured or calculated. In Viscozyme^{TM}L and *Piromyces* sp. enzyme only enzymatic protein accounted for the amount of protein measured in these fractions. This hinders a correct comparison of specific enzymatic activities of and specific oil disclosure by the different preparations. The figures in Table 2 show a tenfold lower specific oil disclosure in artificial rumen fractions compared to *Piromyces* sp. enzyme. However, if 10 % of the biomass protein in culture fractions consists of cell wall degrading enzymes the specific activities would be almost identical. No specific oil disclosing activity was induced by loading the artificial rumen reactors with plant derived material instead of filter paper cellulose (100 % cellulose). The activity profile of several polysaccharide-degrading enzymes in these reactors was also similar (Chapter 5).

The use of extracellular cellulolytic enzymes of isolated anaerobic fungi, bacteria or protozoa with high cell wall degrading capacities might be a better alternative than intact artificial rumen samples to improve oil extraction. Especially anaerobic fungi may have important biotechnological applications. The extracellular cellulolytic enzymes of these organisms can easily be concentrated and used for extraction purposes (Teunissen, pers.comm.). Studies on the growth of and the cellulolytic enzymes produced by these organisms and on cocultures with methanogenic bacteria are in progress (Teunissen et al, 1991a; 1991b; 1992).

This study has demonstrated that the use of intact artificial rumen fractions gave relatively high yields in oil extraction. The use of intact microorganisms and concentration problems, however, diminish the usefulness of these artificial rumen fractions. Enzymes from rumen micro-organisms involved in fibre breakdown might be better suited for extraction purposes.

DEGRADATION OF L-5-VINYLOXAZOLIDINE-2-THIONE (VOT), A GOITROGENIC PRODUCT FROM RAPESEED, BY RUMEN MICROORGANISMS

SUMMARY

An *in vitro* rumen system was used to test the effect of L-5-vinyloxazolidine-2-thione (VOT), a toxic hydrolysis product from glucosinolates found in rapeseed, on rumen performance and to test the ability of rumen microorganisms to degrade this compound. VOT was degraded efficiently (60 - 70 %) in sheep and cow artificial rumen systems, when rapeseed was fed batchwise at 20 % (w/w) of the diet. When added continuously, in concentrations up to 4 mM with the influent buffer, it was completely degraded in a sheep artificial rumen system. The effects of the compound on rumen performance were minimal. In batch incubations mixed rumen microorganisms were able to degrade purified VOT (2 mM) completely.

INTRODUCTION

Rapeseed meal, containing up to 40 % (w/w) protein, is a well established protein feedstuff in livestock industry. The use of this oilseed meal, however, is limited especially by the presence of a group of antinutritional compounds called glucosinolates and furthermore by the presence of tannins and the high fibre content (Fenwick, 1984).

The presence of glucosinolates in crops intended for animal consumption causes serious problems (Clandinin & Robblee, 1981; Thomke, 1981). The most common problem is due to the pungency of the enzymatic hydrolysis products of glucosinolates which decreases the overall palatability of the diet (Fenwick, 1984). Other problems linked to the presence of glucosinolates include goitrogenicity, hepatoxicity and nephrotoxicity (Fenwick et al,1983). Feeding poultry with rapeseed meal is associated with the development of leg abnormalities and liver haemorrhage (Fenwick & Curtis, 1980). Racemic goitrin (at levels resulting from an equivalent of 30 % (w/w) rapeseed meal in the diet) has been shown to depress growth and enlarge the thyroids of chickens (Fenwick & Curtis, 1980). With ruminants and swine, palatability problems are most frequently encountered, resulting from the formation of volatile isothiocyanates (Fenwick, 1984). One of the formed hydrolysis products, 5-vinyloxazolidine-2-thione (VOT), is intensely bitter (Fenwick & Griffiths, 1981).

All investigated species of the Cruciferae family contain one or more glucosinolates. Members of this family are rapeseed (*Brassica napus, B. campestris*), cole crops (*B. oleraceae*), mustards (*B. nigra, B. hirta*) and horseradish (*Armoracia* spp) (VanEtten & Tookey, 1979). Of the nearly 100 known naturally occurring glucosinolates 15 - 20 have been found in the genus *Brassica*. Their qualitative and quantitative composition depends on such factors as species, age of plant and part of plant examined (Fenwick et al, 1983). The physiological responses encountered when plant or products of plants from the Cruciferae family are fed to animals and humans are due to the properties of hydrolysis products; the glucosinolates themselves are biologically inactive (Clandinin & Robblee, 1981; Thomke, 1981; Fenwick & Heaney, 1983).

This hydrolysis is catalysed by an enzyme, myrosinase (thioglucoside hydrolase EC 3:2:3:1), which is found in all plants containing glucosinolates. In intact tissue

myrosinase is kept separated from its substrate in myrosin cells (Thangstad et al, 1990). Disruption of plant cells by cutting, cooking or chewing brings about the enzymatic hydrolysis of glucosinolates (Vos & Blijleven, 1988). In rapeseed meal three glucosinolates, progoitrin (2-hydroxy-3-butenyl glucosinolate), gluconapin (3-butenyl-glucosinolate) and glucobrassicanapin (4-pentenyl-glucosinolate) account for almost all the glucosinolates present (Hill, 1979). The hydrolysis of these glucosinolates by myrosinase results in the formation of oxazolidinethiones (goitrin), isothiocyanates, thiocyanates and nitriles depending on hydrolysis conditions (VanEtten & Tookey, 1978; Fenwick et al, 1983). The reaction scheme for the formation of goitrin and nitriles from progoitrin is given in Fig. 1. The formation of nitriles from progoitrin predominates at low pH, the formation of goitrin at neutral or higher pH and with old or heat treated meal (Tapper & Reay, 1973).

Vinyloxazolidine-2-thione (VOT), a hydrolysis product from progoitrin, is toxic for non-ruminants due to its antithyroid and, therefore, goitrogenic action (Allison, 1978). Ruminants appear to be more resistant to this compound (Hill, 1979; Rutkowski, 1971). Virtanen et al (1958; 1963) and Virtanen (1961) reported that goitrogens were not transferred in significant quantities to milk (< 0.05 % of the ingested amount) and suggested that these goitrogens were probably reduced in the rumen and that only a part of the goitrin, ingested or formed in the rumen, was transferred to the blood. Studies of Bachmann et al (1985) demonstrated that not more than 0.1 % of the original progoitrin content of the feed is transferred to the milk in the form of goitrin and Lanzani et al (1974) presented evidence that sheep rumen fluid could degrade progoitrin and goitrin.

The problems with glucosinolates in rapeseed meal have been partly overcome by breeding of new cultivars, which show an improved palatability and can be included in higher amounts in animal diets (Fenwick, 1984). Various types are available : "zero" is low in erucic acid (< 2 %) but still high in glucosinolates (150 - 180 μmol/g meal) (Pusztai, 1989); low glucosinolate cultivars, usually referred to as low glucosinolate (LG) type, include Tower, Regent, Candle and Altex (Clandinin & Robblee, 1981) and contain 30 - 60 μmol glucosinolates per g of meal (Pusztai, 1989); "double zero" rapeseed or Canola is low in both glucosinolates and erucic acid (Daun, 1986b). Despite the availability of these new cultivars the remaining levels of glucosinolates still limit the use of rapeseed meals, especially for pig and poultry feeding (Pusztai, 1989). Industrial processes of detoxification have so far not proved to be very efficient (Pusztai, 1989).

Recently Gijzen et al (1986) developed an artificial rumen system resembling well the *in vivo* rumen. Compared to the *in vivo* system *in vitro* conditions are more standardized and reproducible and enable the testing of possibly toxic compounds, such as glucosinolates and their hydrolysis products, without the danger of animal toxicity. This *in vitro* rumen system was used as a model system to investigate the effect of VOT on rumen performance and the possible role of rumen microorganisms in the degradation of this compound.

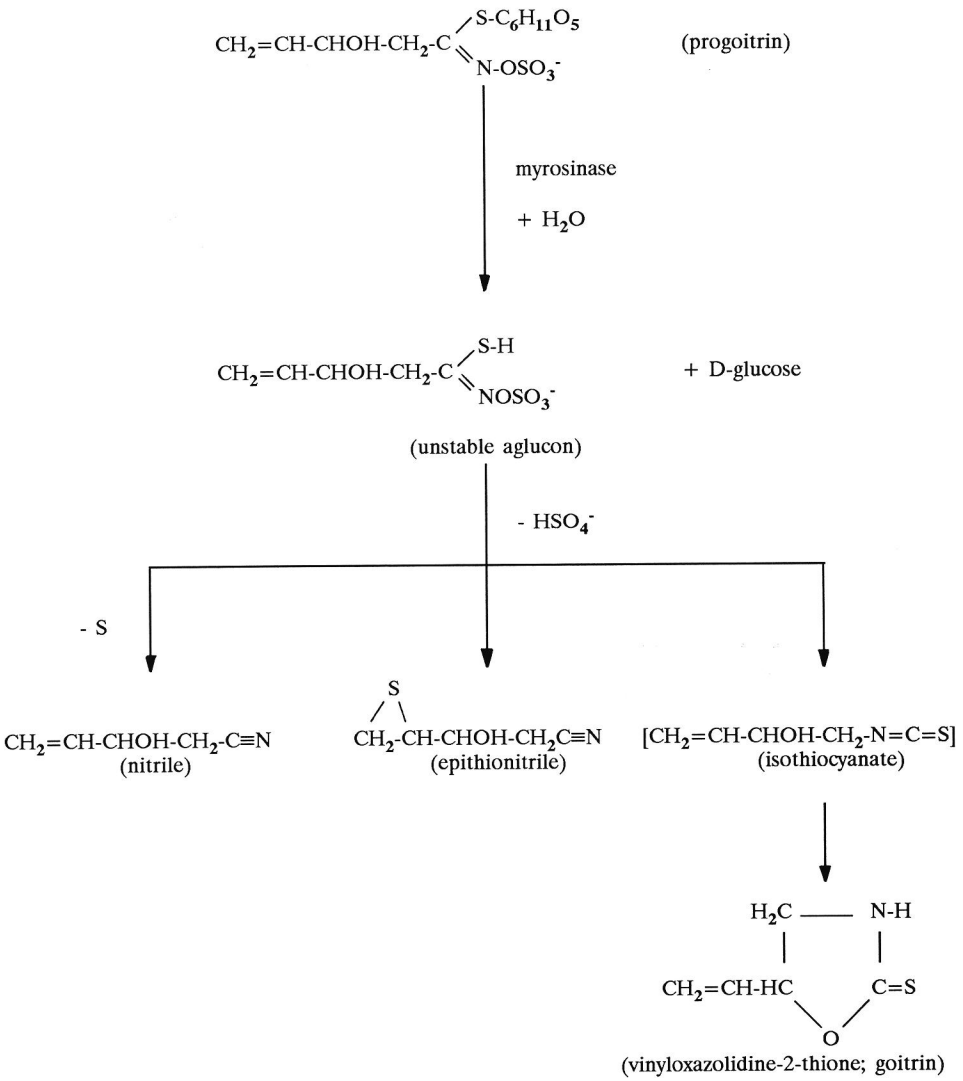


Fig. 1. Products of myrosinase hydrolysis of 2-hydroxy-3-butenylglucosinolate (progoitrin) (VanEtten & Tookey, 1979).

MATERIALS AND METHODS

Digester feeds

Hay and concentrate were a gift from CeHaVe (Veghel, The Netherlands), rapeseed (low glucosinolate quality) was purchased from Speelmans oliefabriek (Rotterdam, The Netherlands).

Rumen buffer consisted of mineral buffer according to Rufener et al (1963) supplemented with 28 mM NH_4HCO_3 and trace elements according to Vishniac & Santer (1957).

The operation of the digesters and the analytical methods are described in Chapter 2. The chemical composition of the substrates used as digester feeds is listed in Table 1.

Table 1. Chemical composition of the substrates

	TS[a] (%)	VS[b] (% of TS)	Crude cellulose (% of TS)	Crude fibre (% of TS)	Starch (% of TS)
Hay	89.5 ± 0.3	91.7 ± 0.3	29.7	7.6	0
Concentrate	87.8 ± 0.1	91.3 ± 0.3	10.9	18.3	18.9
Rapeseed	93.7 ± 1.7	83.6 ± 1.9	n.d.[c]	n.d.	n.d.

[a] Total Solids
[b] Volatile Solids
[c] Not determined

Preparation of crude VOT extracts

Rapeseed (10.4 g) was homogenized with an Ultra-Turrax Model TP.18/2 (IKA-Werk, Staufen, Germany) for 10 min with 50 ml rumen buffer. The slurry was incubated at 39°C and 100 rpm in a shaking water bath for 1 h to ensure maximal VOT-formation. Slurry treated in this way was used when VOT was fed batchwise.

When VOT was included in the influent buffer of the rumen system, 25 - 300 g rapeseed was homogenized with an Ultra-Turrax Model T.45 in 0.3 - 3 l rumen buffer for 15 - 20 min on ice to prevent denaturation of myrosinase due to heat production during homogenisation. After incubation at 39°C and 100 rpm for 1 h, the insoluble material and the oil were removed by centrifugation at 10,000 x g for 30 min; after removal of the floating oil-layer the supernatant was taken and filtered through filter paper, then diluted to the desired concentration of VOT (0.05 - 4 mM) in rumen buffer and used as influent for the artificial rumen system. The VOT-containing influent did

not contain any oil. The influent contained considerable amounts of protein and volatile solids (VS) when higher VOT-concentrations (1.4 - 4.25 mM) were tested since crude rapeseed extracts were used. When 150 g rapeseed was milled in 1 l buffer the VS and protein content amounted to 26 ± 2 and 15 ± 2 $g.l^{-1}$, respectively. The protein content was determined with the bicinchoninic acid protein assay kit from Sigma with bovine serum albumin as a standard.

Purification of VOT

Purified L-5-vinyloxazolidine-2-thione (VOT) was isolated from rapeseed by following the procedure described by Astwood et al (1949) and Elfving (1980). The procedure was slightly modified. Rapeseed (350 g) was homogenized with an Ultra-Turrax in 2 l demineralized water during 10 min. After adding 1 l of demineralized water the slurry was heated to 65°C and kept at that temperature for 15 min to ensure maximal VOT-formation by endogenous myrosinase. The slurry was centrifuged (10 min; 8,000 x g) and the supernatant was filtrated through filter paper. To the residue of the centrifugation step 1 l demineralized water was added, the slurry was then heated to 65°C for 15 min, centrifuged and filtrated again. The combined extracts were concentrated by evaporation to form a brown syrup-like solution. VOT was recovered from the concentrate by five extractions with 400 ml of diethylether. To the combined ether-extracts (± 2 l) 60 ml of a saturated sodium bicarbonate solution was added. After thorough mixing the water phase was removed and 60 ml 2 M sodium hydroxide was added to the ether-extracts, mixed thoroughly again, after which the water phase was removed. The pH of the combined water phases, containing the VOT, was adjusted to 8.5 - 9 by gassing with CO_2. The water solution was extracted five times with 60 ml diethylether. The diethylether fraction was evaporated and the yellow oily residue was shaken with charcoal in 50 ml redistilled water and filtrated. The filtrate was vacuum-dried to a yellowish, oily residue. This residue was dissolved in dried dichloromethane and purified by "flash"-chromatography (Still et al, 1978; Hanson & Smith, 1986). The stationary phase was silicagel 60H (Merck). A pressure of 1.5 - 2.0 bar was used to obtain the necessary flow rate. The column length was approximately 15 - 20 cm, column diameters varied between 2 and 5 cm. The mobile phase consisted of a mixture of hexane and ethylacetate (1 : 1). The fractions containing VOT (detectable by UV-absorption) were combined and evaporated. The residue was dissolved in a small volume of dried diethylether, cooled and crystallized. The yield of 2.145 g/kg is somewhat lower than the yield of 3.171 g/kg reported by Elfving (1980), using a comparable method. Crystals were stored in the dark at -20°C under nitrogen.

Characterization of VOT

VOT was isolated as colourless crystals. The melting point and the specific rotation ($[\alpha]_D^{31}$) of the crystals were 48 - 50°C and -71.2°, respectively, comparable to the values reported by Astwood et al (1949) and Elfving (1980) (melting point 49 - 50°C, specific

rotation, $[\alpha]_D^{31}$, -71°). The UV-absorption spectrum of the purified VOT in aqueous solution is given in Fig 2. The maximum absorption is found at 242 nm. The IR-spectrum, the mass spectrum, with a molecular peak at m/e 129, and the NMR-spectrum (Fig. 2) are in complete agreement with those reported by Elfving (1980).

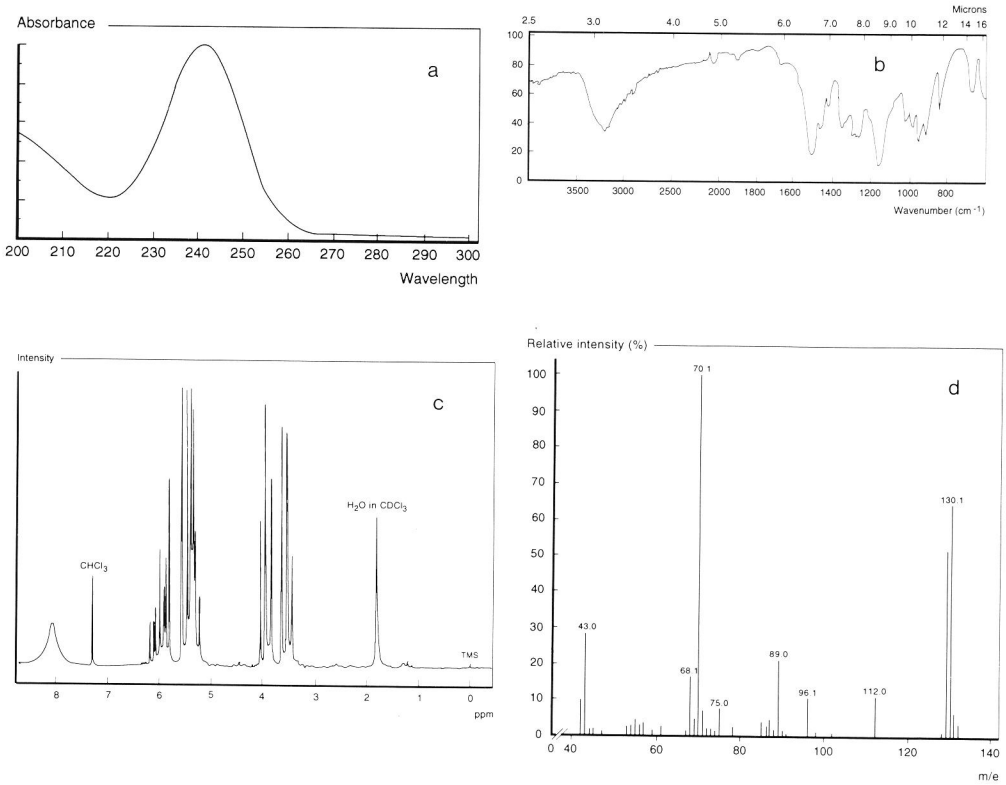


Fig. 2. UV-absorption spectrum (a), the IR-spectrum (b), the NMR spectrum (c) and the mass spectrum (d) of VOT.

VOT-analysis

VOT-containing effluent samples from artificial rumen reactors and samples from batch incubations were heated at 100°C for 10 min to denature proteins. After cooling

the samples were centrifuged at 48,000 x g for 30 min. The supernatant was diluted with two volumes methanol-borate buffer (85 ml 0.05 M $Na_2B_4O_7.10H_2O$ mixed with 15 ml 0.1 M HCl, adjusted to pH 9.0 and mixed with methanol in the ratio 85 : 15). After filtering (45 μm) the samples were used for HPLC-analysis. Samples were stored at -20°C.

To determine the VOT content of the fibre containing fraction in the system, a homogeneous sample from the system (600 ml) was centrifuged (45 min; 13,000 x g). The supernatant was sampled and the residue was resuspended in the original volume of demineralised water and centrifuged. This washing procedure was repeated once more. All supernatants were sampled. A sample of the remaining residue was extracted with diethylether (4 times), the diethylether was removed by evaporation and the residue was dissolved in an 1 % NH_3 (v/v) solution (adapted from Bachmann et al, 1985). The obtained sample was treated as described above.

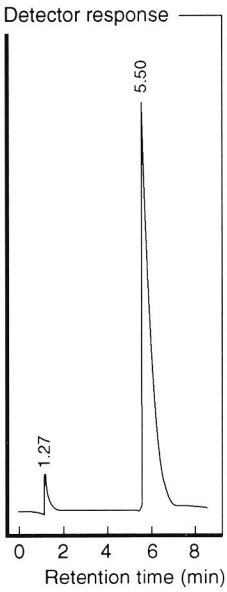


Fig. 3. HPLC-chromatogram of purified VOT.

HPLC-analysis

HPLC was performed with a Perkin-Elmer LC-pump (series 410 BIO) on a Lichrocart 125-4 Lichrospher 100 RP 18 (5 μm) column (Merck) at ambient temperature. The eluent was monitored at 242 nm on a Perkin-Elmer (LC 90 BIO) UV detection unit equipped with a Perkin Elmer (LC 100) Integrator. The column was eluted with a methanol-redistilled water mixture (10 : 90) at 1 ml/min. The purified VOT-crystals, used as a standard, dissolved in methanol or redistilled water showed a single peak with a retention time of 5.5 min (Fig. 3). The detector response was linear in the range of 0.1 - 30 μg/l VOT.

Chemicals

All chemicals used in the extraction procedures and sample preparation were of analytical grade. Dichloromethane was dried by distillation from phosphorus pentoxide

and diethylether was pre-dried on calcium chloride and then distilled from sodium hydride. Methanol used for HPLC-analysis was of HPLC-grade (Baker).

Microbiological methods

To test the degradation of purified VOT by mixed rumen microorganisms the mineral solutions from Medium 10 described by Caldwell & Bryant (1966) were used. Titanium(III)citrate (0.15 mM) was used as a reductor. The medium was supplemented with trace elements according to Vishniac & Santer (1957) and resazurin (1 mg/l). All medium ingredients were filter sterilized. Incubations were performed under an atmosphere of CO_2 or H_2/CO_2 (80 % : 20 %) in 10 ml serum bottles with butyl rubber stoppers at 39°C and 100 rpm. A 10 % inoculum was used.

RESULTS

Degradation of VOT by rumen microorganisms

In order to establish the potential role of rumen microorganisms in the degradation of VOT, artificial rumen reactors were loaded with a rapeseed containing diet. Fermenters were inoculated with strained rumen fluid from a fistulated sheep or cow. Degradation efficiency was studied at a loading rate of 27.9 g $VS.l^{-1}.d^{-1}$ and a hay - concentrate ratio of 1 : 2 for diets without rapeseed. For diets including rapeseed a loading rate of 27.9 g $VS.l^{-1}.d^{-1}$ was used and a hay - concentrate - rapeseed ratio of 1.00 : 1.33 : 0.56 was used, equivalent to a feed with 20 % rapeseed. The VOT content of the homogenized rapeseed slurry amounted to 370 ± 35 μmol per 10.4 g rapeseed milled (n = 17). A solid retention time of 72 h was employed for all reactors. The hydraulic retention times of the reactors inoculated with either cow or sheep rumen fluid were kept at 10.0 ± 1.0 h and 12.5 ± 1.0 h, respectively. Efficiency of degradation, pH, volatile fatty acid (VFA) production, biogas production and VOT degradation were determined at steady state conditions as described in Chapter 2. The results are shown in Table 2 and 3. The degradation of VOT was determined by measuring the amount of VOT in the rapeseed slurry administered with the feed, in the total collected effluent and in the effluent of the reactor 24 h after administering the feed to determine the residual amount of VOT in the system.

VS degradation efficiency was hardly affected by the rapeseed supplementation of the diet. In contrast, biogas and methane production were lowered significantly by the addition of rapeseed to the system. The pH was similar in the sheep artificial rumen reactor and slightly higher in the cow artificial rumen reactor when rapeseed was included in the diet. Ciliated protozoa disappeared from the system when an oil-containing rapeseed mixture was added to the system; a similar result was described in Chapter 3. VFA production was identical for both diets in a sheep artificial rumen reactor. In a cow artificial rumen reactor VFA production was slightly lower when

rapeseed was added to the diet. The production of propionic acid and C_5 - C_7 acids was enhanced whereas the production of acetic acid was lowered when rapeseed was added to the diet. VOT was degraded efficiently in both reactor types.

The fibre fraction, which was removed from the system every day, was washed and extracted with diethylether to check if VOT attached to fibres. No VOT was present in the extracts of any of the supernatants; in the extract from the fibre fraction, suspended in 1 % NH_3 a range 0 - 55 μmol VOT.l^{-1} was found. VOT-degradation efficiencies were corrected for this amount.

Table 2. Steady state degradation of a hay-concentrate diet in different artificial rumen systems

	SHEEP	COW
VS degradation (%)	68 ± 5	70 ± 5
VFA production (mmol.l^{-1}.d^{-1})	138 ± 16[a]	142 ± 10[a]
Biogas production ($l.l^{-1}.d^{-1}$)	5.9 ± 0.7	5.5 ± 0.5
Methane production ($l.l^{-1}.d^{-1}$)	1.1 ± 0.1	1.1 ± 0.1
pH	6.6 ± 0.1	6.6 ± 0.1

[a] Mean molar ratio of VFA
acetic acid : propionic acid : butyric acid : C_5 - C_7 acids = 62 : 20 : 13 : 5 (sheep) and 63 : 19 : 14 : 5 (cow)

Table 3. Steady state degradation of a hay-concentrate diet supplemented with milled rapeseed in different artificial rumen systems

	SHEEP	COW
VS degradation (%)	65 ± 8	64 ± 10
VFA production (mmol.l^{-1}.d^{-1})	136 ± 10[a]	120 ± 10[a]
Biogas production ($l.l^{-1}.d^{-1}$)	3.8 ± 0.5	3.5 ± 0.6
Methane production ($l.l^{-1}.d^{-1}$)	0.4 ± 0.1	0.3 ± 0.1
pH	6.7 ± 0.1	6.9 ± 0.1
VOT degradation (%)	72 ± 8	62 ± 7

[a] Mean molar ratio of VFA
acetic acid : propionic acid : butyric acid : C_5 - C_7 acids = 52 : 24 : 12 : 11 (sheep) and 54 : 23 : 11 : 11 (cow)

Adaptation to rapeseed loading

In order to establish if rumen microorganisms are able to degrade VOT instantly, or if an adaptation period is required, the performance of an artificial rumen system was followed immediately after a diet change. An artificial rumen reactor inoculated with sheep rumen fluid was loaded with a hay-concentrate diet for 21 days and changed to a diet including rapeseed at day 0. The hydraulic retention time was kept at 13.4 ± 0.4 h and the solid retention time at 72 h. VS and VOT degradation efficiencies and fermentation parameters are given in Table 4 and 5.

Table 4. Degradation efficiency and fermentation parameters in an artificial rumen system during adaptation to a rapeseed containing diet.

	Degradation		Production			
	VS (%)	VOT (%)	VFA ($mmol.l^{-1}.d^{-1}$)	biogas ($l.l^{-1}.d^{-1}$)	methane ($l.l^{-1}.d^{-1}$)	pH
preinc.[a]	72	n.d.[b]	151	6.0	1.1	6.7
preinc.	68	n.d.	151	5.5	1.1	6.7
day 0	72	78	153	5.9	1.4	6.6
day 1	70	74	147	4.8	1.1	6.8
day 5	62	67	156	3.5	0.6	6.7
day 6	62	66	189	3.2	0.6	6.8
day 13	60	78	178	3.3	0.5	6.8
day 14	59	78	164	3.1	0.5	6.8

[a] Preincubation period, 1 week before rapeseed loading was started at day 0
[b] Not determined

Table 5. Mean molar ratio of VFA (%), produced during the adaptation to a rapeseed containing diet

	A	P	B	C_5-C_7 acids
preinc	59	19	19	4
day 0/1	59	19	17	5
day 5/6	45	42	8	5
day 13/14	46	41	7	6

Abbreviations : A acetic acid, P propionic acid, B butyric acid

VOT was degraded efficiently from the first day it was added to the system. The most significant effects of rapeseed supplementation to the diet were a reduction of biogas and methane production and the enhancement of propionic acid production. The production of acetic and butyric acid was lowered significantly.

Continuous addition of VOT with the influent buffer

When VOT is fed batchwise complete degradation of this compound is not possible, since effluent is removed continuously taking out VOT at a high concentration in the period right after feeding. To determine the VOT degrading capacity of the artificial rumen system, a crude VOT preparation (rapeseed extract) mixed with the influent buffer was added continuously to the system. Concentrations ranging from 0.05 to 4.0 mM of VOT were tested. The concentration of VOT in the influent buffer was increased after 5 - 14 days. Degradation efficiencies were determined after 3 - 10 days adaptation. An artificial rumen system inoculated with sheep rumen fluid was used. The hydraulic retention time was kept at 12.5 ± 1.0 h and the solid retention time at 72 h during all experiments. Cultures were loaded with a hay-concentrate diet. A loading rate of 27.9 g $VS.l^{-1}.d^{-1}$ and a hay : concentrate ratio of 1 : 2 was applied for cultures with VOT influent concentrations ranging from 0.05 to 0.75 mM, while for concentrations ranging from 1.5 to 4.0 mM a loading rate of 22.2 g $VS.l^{-1}.d^{-1}$ was applied with a hay : concentrate ratio of 1.0 : 0.9 to prevent acidification of the system. Degradation efficiencies and fermentation parameters are shown in Table 6.

Table 6. Effect of increasing VOT load on VS and VOT degradation efficiency, VFA, biogas and methane production and pH.

	Degradation		Production			
[crude VOT] (mM)	VS (%)	VOT (%)	VFA ($mmol.l^{-1}.d^{-1}$)	biogas ($l.l^{-1}.d^{-1}$)	methane ($l.l^{-1}.d^{-1}$)	pH
0	73	n.d.[a]	167 ± 15[b]	5.5 ± 0.3	1.1 ± 0.1	6.7 ± 0.1
0.05	72	> 99	189 ± 8	5.5 ± 0.1	1.1 ± 0.1	6.7 ± 0.1
0.60	77	> 99	190 ± 22	5.4 ± 0.5	1.0 ± 0.1	6.6 ± 0.1
0.75	77	> 99	197 ± 6	5.5 ± 0.5	1.1 ± 0.1	6.5 ± 0.1
1.40	73	> 99	203 ± 9	5.5 ± 0.1	1.0 ± 0.1	6.7 ± 0.1
2.20	80	> 99	273 ± 10	4.7 ± 0.8	0.7 ± 0.2	6.5 ± 0.1
4.25	82	99	301 ± 20	4.3 ± 1.0	0.1 ± 0.1	6.4 ± 0.1

[a] Not determined

[b] Mean molar ratio of VFA
acetic acid : propionic acid : butyric acid : C_5 - C_7 acids = 64 : 17 : 14 : 6 for all concentrations except for 2.2 and 4.25 mM, mean molar ratio for these concentrations 51 : 23 : 16 : 13

Adding a crude rapeseed extract to the influent buffer of the artificial rumen system had little effect on VS degradation. The production of VFA increased significantly at higher VOT-concentrations, even though the amount of VS added to the system as solid substrate was lowered from 27.9 to 22.2 g $VS.l^{-1}.d^{-1}$. The amount of VS added with the influent buffer, however, increased considerably. Methane production was affected at high VOT-concentrations and practically stopped at 4 mM VOT. Ciliated protozoa disappeared from the system at high VOT-concentrations (≥ 2 mM). VOT degradation was very efficient at all loading rates tested.

Degradation of purified VOT by mixed rumen microorganisms in batch cultures

The degradation of purified VOT (2 mM) in buffer under CO_2 or H_2/CO_2 atmosphere was tested. When fresh rumen fluid was used as an inoculum (10 % v/v) VOT was degraded completely within 24 hours. When samples from the artificial rumen reactor loaded with 0.75, 2.20 or 4.25 mM VOT in the influent buffer were used as inoculum the VOT was also completely degraded within the same period. No VOT degradation was measured when artificial rumen samples were filter sterilised before incubation.

DISCUSSION

This study made clear that no differences were seen between artificial rumen reactors inoculated with sheep or cow rumen fluid when reactors were loaded with a hay-concentrate diet (Table 2). This is in agreement with feeding trials *in vivo*. On highly digestible diets sheep digest more than cattle, on poor quality diets cattle are superior. At 68 - 70 % digestibility the performance of both animals is comparable (Lindgren, 1987). As shown in Chapter 3 the addition of rapeseed oil had a significant effect on different fermentation parameters. However, when the amount of rapeseed in the diet did not exceed 20 % (w/w) no significant effect on VS degradation or VFA-production was seen. This again is in agreement with feeding trials with cattle *in vivo*, which indicate that up to 25 % rapeseed meal can be included in the diet without adverse effects on milk yield or composition, feed intake and general health of the animal (Sharma et al, 1977; Papas et al, 1978). The effect of the oil added to the system was limited to the disappearance of the ciliated protozoa and a reduction of the amount of methane produced. *In vivo* such a reduction of the numbers of ciliated protozoa following the addition of linseed, soybean or safflower oil to the rumen as part of the diet was also found by Ikwuegbu & Sutton (1982), Broudiscou et al (1988) and O'Kelly & Spiers (1990). The reduction in methane formation was probably connected with the higher amount of propionic acid and the lower amounts of acetic and butyric acid formed. This effect was also seen when propionic acid enhancers like monensin were used (Van Nevel & Demeyer, 1988). Schelling (1984) showed that an increase in propionic acid production in the rumen resulted in a protein-sparing effect, as the

animal used less amino acids for gluconeogenesis under these circumstances. The increased formation of C_5 - C_7 acids upon inclusion of rapeseed in the diet was due to the addition of extra protein to the system.

The presence of glucosinolates in rapeseed meal limits the use of this meal in animal feedstuffs and human foods. Myrosinase, present in rapeseed, is usually inactivated by heat treatment or cooking. This leads to the ingestion of intact glucosinolates (Fenwick & Heaney, 1983). However, glucosinolates can also be hydrolysed after ingestion. When laying hens were fed intact glucosinolates, about 50 % of the administered dose was recovered in faeces and urine as intact glucosinolates. Only 1 - 2 % of the administered dose was found as hydrolysis products of these glucosinolates. On basis of these results Freig et al (1987) suggest the presence of myrosinase activity in the intestinal tract of these hens. Oginsky et al (1965) already demonstrated myrosinase activity in a variety of bacteria, particularly *Paracolobacterium*, which commonly inhabits the intestinal tract of mammals. Marangos & Hill (1974) and Nugon-Baudon et al (1988) also reported the degradation of glucosinolates by bacteria in the intestinal tract of poultry and rats.

In this study ruminal diets containing VOT were applied. VOT degradation was efficient in both sheep and cow artificial rumen reactors. In the experiments, in which VOT was fed batchwise complete degradation of VOT was not possible since VOT was removed continuously with the effluent, especially in the period right after feeding. The slightly lower VOT degradation efficiency in a cow artificial rumen reactor can be explained by the lower hydraulic retention time. When a crude VOT preparation was added continuously in the influent buffer, VOT was degraded completely up till concentrations of 4.25 mM. The VOT degradation capacity of the artificial rumen reactor amounts to at least 8 $mmol.l^{-1}.d^{-1}$. The decrease of methane production and the increase of VFA production when higher VOT concentrations ($\geq$ 1.4 mM) were applied were most likely caused by the increasing VS and protein content of the influent buffer since a crude preparation of VOT was used. VS degradation increased slightly at higher VOT-concentrations because less VS was fed with the diet and large amounts of easily degradable VS were added with the influent buffer.

The present study shows that considerable amounts of goitrin can be degraded in the artificial rumen reactor. Lanzani et al (1974) already showed that both progoitrin and goitrin can be degraded by rumen fluid *in vitro*. In a subsequent study VanEtten et al (1977) investigated the effect of rations containing up to 10 % processed crambe seed meal. The meal used contained varying amounts of progoitrin, unsaturated nitriles and goitrin. No detectable levels of glucosinolates or breakdown products as nitriles or goitrin were found in the body tissues of cattle fed these rations. VT (5-vinyl-1,3-thiazolidine-2-one), an isomer of goitrin found by Lanzani et al (1974) in incubation mixtures of sheep rumen fluid with progoitrin, was not detected in the body tissues. Nitriles, produced by autolysis from glucosinolates in swedes (*Brassica napus* var *napobrassica*), were also degraded by rumen fluid *in vitro* (Forss & Barry, 1983). In the same set of experiments, however, nitriles formed by autolysis from kale (*Brassica oleracea* var. *acephala*) appeared stable for more than 24 hours when incubated with rumen fluid *in vitro*. The degradation of VOT in the artificial rumen may explain the observed resistance of ruminants to the inclusion of rapeseed meal in their diets.

Isolation procedures of the microorganisms responsible for the VOT degradation observed in the artificial rumen system are in progress.

In conclusion, this study shows that L-5-vinyloxazolidine-2-thione can be degraded by rumen microorganisms *in vitro*. Effects of this toxic compound on the performance of the artificial rumen system were minimal. The observed effects could be explained by the use of a crude preparation. The artificial rumen system may be used to predict the effect of (toxic) dietary ingredients on rumen performance *in vivo*.

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REFERENCES

Akin DE (1980)
Evaluation by electron microscopy and anaerobic culture of types of rumen bacteria associated with digestion of forage cell walls. Appl Environ Microbiol 39:242-252
Akin DE, Amos HE (1979)
Mode of attack on orchardgrass leaf blades by rumen protozoa. Appl Environ Microbiol 37:332-338
Akin DE, Benner R (1988)
Degradation of polysaccharides and lignin by ruminal bacteria and fungi. Appl Environ Microbiol 54:1117-1125
Allison MJ (1978)
The role of ruminal microbes in the metabolism of toxic constituents from plants. In : Keeler RF, van Kampen KR, James CF (eds), Effects of poisonous plants on livestock. Academic Press, New York
Allison MJ, Hammond AC, Jones RJ (1990)
Detection of ruminal bacteria that degrade toxic dihyroxypyridine compounds produced from mimosine. Appl Environ Microbiol 56:590-594
Amos HE, Akin DE (1978)
Rumen protozoal degradation of structurally intact forage tissues. Appl Environ Microbiol 36:513-522
Anjou K (1972)
Manufacture of rapeseed oil and meal. In : Appelqvist LA, Ohlson R (eds), Rapeseed : cultivation, composition, processing and utilization. Elsevier Amsterdam, London, New York
Anonymous (1975)
Standard Methods for the examination of water and waste water (3rd edn.) American Public Health Association, New York.
Arambel MJ, Bartley EE, Dennis SM, Riddell DO, Camac JL, Higginbotham JF, Simons GG, Dayton AD (1986)
Effect of toasted soybean meal with or without niacin on rumen fermentation, passage rate of duodenal digesta and digestibility of nutrients. Nutrition Reports International 34:1011-1021
Astwood EB, Greer MA, Ettlinger MG (1949)
L-5-vinyl-2-thiooxazolidone, an antithyroid compound from yellow turnip and from brassica seeds. J Biol Chem 181:121-131
Bachmann M, Theus R, Lüthy J, Schlatter C (1985)
Vorkommen von goitrogenen Stoffen in Milch. Z Lebensm Unters Forsch 181:375-378
Bailey RW (1973)
Structural carbohydrates. In : Butler CW, Bailey RW (eds), Chemistry and biochemistry of herbage vol I, Academic Press London-New York
Baldwin RL (1984)
Digestion and metabolism of ruminants. BioScience 34:244-249
Bauchop T, Elsden SR (1960)
The growth of micro-organisms in relation to their energy supply. J Gen Microbiol 23:457-469
Becker KW (1971)
Processing of oilseeds to meal and protein flakes. J Am Oil Chem Soc 48:299-304
Bell JM (1984)
Nutrients and toxicants in rapeseed meal : a review. J Am Oil Chem Soc 58:996-1010
Bengtsson L, Von Hofsten A, Lööf B (1972)
Botany of rapeseed. In : Appelqvist LA, Ohlson R (eds), Rapeseed : cultivation, composition, processing and utilization. Elsevier Amsterdam, London, New York
Benner R, Hodson RE (1985)
Thermophilic anaerobic biodegradation of [^{14}C]lignin, [^{14}C]cellulose, and [^{14}C]lignocellulose preparations. Appl Environ Microbiol 50:971-976
Benner R, Maccubbin AE, Hodson RE (1984)
Anaerobic biodegradation of the lignin and polysaccharide components and synthetic lignin by sediment microflora. Appl Environ Microbiol 47:998-1004
Berger LL (1988)
Effects of diet composition on rumen fermentation. Animal & Plant Sciences 1:178-182
Bhatnagar S, Johari BN (1987)
Microbial enzymes in the processing of oilseeds. Curr Sci 56:775-776

Bisaria VS, Ghose TK (1981)
Biodegradation of cellulosic materials : substrates, microorganisms, enzymes and products. Enzyme Microb Technol 3: 90-104
Bradford MM (1976)
A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 72:248-254
Brock FM, Forsberg CW, Buchanan-Smith JG (1982)
Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. Appl Environ Microbiol 44:561-569
Brooks JR, Morr CV (1984)
Phytate removal from soybean proteins. J Am Oil Chem Soc 61:1056-1059
Broudiscou L, van Nevel CJ, Demeyer DI, Jouany JP (1988)
Addition d'hydrolysat d'huile de soja dans la ration de mouton Effet sur la dégradation *in sacco* de la paille de la cellulose. Reprod Nutr Dévelop 28:159-160
Bryant MP (1973)
Nutritional requirements of the predominant rumen cellulolytic bacteria. Fed Proc 32:1809-1813
Caldwell DR, Bryant MP (1966)
Medium without rumen fluid for non-selective enumeration and isolation of rumen bacteria. Appl Microbiol 14:794-801
Chandler JA, Jewell WJ, Gosset JM, van Soest PJ, Robertson JB (1980)
Predicting methane fermentation biodegradability. Biotechnol Bioeng Symp 10:93-107
Chen M, Wolin MJ (1979)
Effect of monensin and lasalocid-sodium on the growth of methanogenic and rumen saccharolytic bacteria. Appl Environ Microbiol 38:72-77
Cheng KJ, Akin DE, Costerton JW (1977)
Rumen bacterial interaction with particulate dietary components and response to dietary variation. Fed Proc 36:193-197
Chesson A, Forsberg CW (1988)
Polysaccharide degradation by rumen microorganisms. In : Hobson PN (ed), The rumen microbial ecosystem. Elsevier Applied Science, London-New York
Chesson A, Stewart CS, Dalgarno K, King TP (1986)
Degradation of isolated grass mesophyll, epidermis and fibre cell walls in the rumen and by cellulolytic rumen bacteria in axenic culture. J Appl Bacteriol 60:327-336
Citron A, Breton A, Fonty G (1987)
Rumen anaerobic fungi. Bull Inst Pasteur 85:329-343
Clandinin DR, Robblee AR (1981)
Rapeseed in animal nutrition : II Nonruminant animals. J Am Oil Chem Soc 58:682-686
Clarke RTJ (1977)
Protozoa in the rumen ecosystem. In : Clarke RTJ, Bauchop T (eds), Microbial ecology of the gut. Academic Press Ltd, London
Coleman GS (1985)
The cellulase content of 15 species of entodiniomorphid protozoa, mixed bacteria and plant debris isolated from the ovine rumen. J Agric Sci Camb 104:349-360
Coleman GS (1986)
The metabolism of rumen ciliate protozoa. FEMS Microbiol Rev 39:321-344
Coughlan MP (1985)
The properties of fungal and bacterial cellulases with comment on their production and application. Biotechnology and Genetic Engineering Reviews 3:39-109
Counotte GHM (1981)
Regulation of lactate metabolism in the rumen. Thesis, Rijksuniversiteit Utrecht, The Netherlands
Daun JK (1986a)
Erucic acid levels in western Canadian Canola and rapeseed. J Am Oil Chem Soc 63:321-324
Daun JK (1986b)
Glucosinolate levels in western canadian rapeseed and canola. J Am Oil Chem Soc 63:639-643

Dawson KA, Allison MJ (1988)
Digestive disorders and nutritional toxicity. In : Hobson PN (ed), The rumen microbial ecosystem. Elsevier Applied Science, London-New York
Demeyer, DI (1981)
Rumen microbes and digestion of plant cell walls. Agric Env 6:295-337
Demeyer DI, Van Nevel CJ (1975)
Methanogenesis, an integrated part of carbohydrate fermentation, and its control. In : McDonald IW, Warner ACI (eds), Digestion and metabolism in the ruminant. University of New England Publishing Unit, Armidale, Australia
Dominguez-Bello MG, Stewart CS (1990a)
Degradation of mimosine, 2,3-dihydroxypyridine and 3-hydroxy-4(1H)-pyridone by bacteria from the rumen of sheep in Venezuela. FEMS Microbiol Ecol 73:283-290
Dominguez-Bello MG, Stewart CS (1990b)
Effects of feeding *Canavalia ensiformis* on the rumen flora of sheep, and of the toxic amino acid canavanine on rumen bacteria. System Appl Microbiol 13:388-393
Doreau M, Michalet-Doreau B (1988)
Dégradibilité *in sacco* de la matière sèche et des constituants pariétaux dans le rumen. Effet de l'apport de matières grasses dans la ration de la vache latière. Reprod Nutr Dévelop 28:157-158
Elfving S (1980)
Studies on the naturally occurring goitrogen 5-vinyl-2-thiooxazolidone. Metabolism and antithyroid effect in the rat. Ann Clin Res Suppl 12:5-47
Erickson DR (1983)
Soybean oil : update number one. J Am Oil Chem Soc 60:351-356
Ettlinger MG, Kjaer A (1968)
Sulphur compounds in plants. Recent Adv Phytochem 1:59-144
Eveleigh DE (1987)
Cellulases, a perspective. Phil Trans R Soc Lond A321:435-447
Fenwick GR (1984)
Rapeseed as an animal feedingstuff - The problem and analysis of glucosinolates. J Assoc Publ Analysts 22:117-130
Fenwick GR, Curtis RF (1980)
Rapeseed meal and its use in poultry diets. A review. Animal Feed Science and Technology 5:255-298
Fenwick GR, Griffiths MM (1981)
The identification of the goitrogen 5-vinyloxazolidine-2-thion (goitrin) as a bitter principle of cooked Brussels sprouts (*Brassica oleraceae* L. var. gemmifera). Z Lebensm Unters Forsch 172:90
Fenwick GR, Heaney RK (1983)
Glucosinolates and their breakdown products in cruciferous crops, foods and feedingstuffs. Food Chem 11:249-271
Fenwick GR, Heaney RK, Mullin WJ (1983)
Glucosinolates and their breakdown products in food and food plants. CRC Crit Rev Fd Sci Nutr 18:123-201
Fisher LJ, Walsh DS (1976)
Substitution of rapeseed meal for soybean meal as a source of protein for lactating cows. Can J Anim Sci 56:233-242
Forss DA, Barry TN (1983)
Observations on nitrile production during autolysis of kale and swedes, and their stability during incubation with rumen fluid. J Sci Food Agric 34:1077-1084
Francis GL, Gawthorne JM, Storer GB (1978)
Factors affecting the activity of cellulases isolated from the rumen digesta of sheep. Appl Environ Microbiol 36:643-649
Freig AAH, Campbell LD, Stanger NE (1987)
Fate of ingested glucosinolates in poultry. Nutr Rep Int 36:1337-1345
Fullbrook PD (1983)
The use of enzymes in the processing of oilseeds. J Am Oil Chem Soc 60:476-480

Garcia Serrato A (1981)
Extraction of oil from soybeans. J Am Oil Chem Soc 58:157-159
Gattinger LD, Duvnjak Z, Khan AW (1990)
The use of canola meal as a substrate for xylanase production by *Trichoderma reesei*. Appl Microbiol Biotechnol 33:21-25
Gijzen HJ, Lubberding HJ, Verhagen FJ, Zwart KB, Vogels GD (1987)
Application of rumen microorganisms for an enhanced anaerobic degradation of solid organic waste materials. Biol Wastes 22:81-95
Gijzen HJ, Zwart KB, van Gelder PT, Vogels GD (1986)
Continuous cultivation of rumen microorganisms, a system with possible applications to the anaerobic degradation of lignocellulosic waste materials. Appl Microbiol Biotechnol 25:155-162
Gijzen HJ, Zwart KB, Verhagen FJ, Vogels GD (1988)
High-rate two-phase process for the anaerobic degradation of cellulose, employing rumen microorganisms for an efficient acidogenesis. Biotechnol Bioeng 31:418-425
Godfrey T (1983)
Industrial applications. In : Godfrey T, Reichelt J (eds), The application of enzymes in industry. Nature Press New York.
Goering HK, van Soest PJ (1970)
Forage fiber analyses. USDA, ARS Agric Handbook no.379:1-20
Gonzalez-Lopez J, Salmeron V, Ramos-Cormenzana A, Silva-Colomer J, Boza J (1990)
Influence of several feeds on bacteria in sheep and goat rumen liquor *in vitro*. Microbios 62:75-81
Halliwell GJ (1957)
Cellulolytic preparations from micro-organisms of the rumen and from *Myrothecium verrucaria*. J Gen Microbiol 17:166-183
Hammond AC, Allison MJ, Williams MJ, Prine GM, Bates DB (1989)
Prevention of leucaena toxicosis of cattle in Florida by ruminal inoculation with 3-hydroxy-4-(1H)-pyridone-degrading bacteria. Am J Vet Res 50:2176-2180
Hanson RW, Smith NG (1986)
Flash chromatography, a recent advance in preparative chromatography. Educ Chem 23:45-46
Harfoot CG, Hazlewood GP (1988)
Lipid metabolism in the rumen. In : Hobson PN (ed), The rumen microbial ecosystem. Elsevier Applied Science, London-New York
Hazlewood GP, Kemp P, Lander D, Dawson RMC (1976)
C_{18} unsaturated fatty acid hydrogenation patterns of some rumen bacteria and their ability to hydrolyse exogenous phospholipid. Br J Nutr 35:293-297
Hegarty MP, Court RD, Christie GS, Lee CP (1976)
Mimosine in *Leucaena leucocephala* is metabolised to a goitrogen in ruminants. Aust Vet J 52:490
Hermansson AM (1986)
Soy protein gelation. J Am Oil Chem Soc 63:658-666
Hespell RB (1988)
Microbial digestion of hemicelluloses in the rumen. Microbiol Sci 5:362-365
Hespell RB, Wolf R, Bothast R (1987)
Fermentations of xylans by *Butyrivibrio fibrisolvens* and other ruminal bacteria. Appl Environ Microbiol 53:2849-2853
Hill R (1979)
A review on the "toxic" effects of rapeseed meals with observations on meal from improved varieties. Br Vet J 135:3-16
Hobson PN (1988)
The rumen microbial ecosystem. Elsevier Applied Science, London-New York
Hobson PN, Wallace RJ (1982a)
Microbial ecology and activities in the rumen : part I. CRC Critical Reviews in Microbiology 9:165-225
Hobson PN, Wallace RJ (1982b)
Microbial ecology and activities in the rumen : part II. CRC Critical Reviews in Microbiology 9:253-320
Hungate RE (1966)
The rumen and its microbes. Academic Press, New York-London

Hungate RE (1988)
The ruminant and the rumen. In : Hobson PN (ed), The rumen microbial ecosystem. Elsevier Applied Science, London-New York
Hutten TJ, de Jong MH, Peeters BPH, van der Drift C, Vogels GD (1981)
Coenzyme M (2-mercaptoethanesulfonic acid)-derivates and their effect on methane production from carbon dioxide and methanol by cell free extracts of *Methanosarcina barkeri*. J Bacteriol 145:27-34
Ikwuegbu OA & Sutton JD (1982)
The effect of varying the amount of linseed oil supplementation on rumen metabolism in sheep. Br J Nutr 48:365-375
Ingalls JR, Sharma HR (1975)
Feeding of Bronowski, Span and commercial rapeseed meals with or without addition of molasses or flavor in rations of lactating cows. Can J Anim Sci 55:721-729
Jones RJ, Lowry JB (1984)
Australian goats detoxify the goitrogen 3-hydroxy-4(1H)pyridone (DHP) after rumen infusion from an Indonesian goat. Experientia 40:1435-1436
Jones RJ, Megarrity RG (1986)
Successful transfer of DHP-degrading bacteria from Hawaiian goats to Australian ruminants to overcome the toxicity of *Leucaena*. Aust Vet J 63:259-262
Josefsson E (1972)
Nutritional value and use of rapeseed meal. In : Appelqvist LA, Ohlson R (eds), Rapeseed : cultivation, composition, processing and utilization. Elsevier Amsterdam, London, New York
Kaufmann W, Hagemeister H, Dirksen G (1980)
Adaptations to changes in dietary composition, level and frequency of feeding. In : Ruckebusch Y, Thivend P (eds), Digestive physiology and metabolism in ruminants. MTP Press Limited, Lancaster, England
Kellens MJ, Goderis HL, Tobback PP (1986)
Biohydrogenation of unsaturated fatty acids by a mixed culture of rumen microorganisms. Biotechnol Bioeng 28:1268-1276
Kopecny J, Wallace RJ (1982)
Cellular location and some properties of proteolytic enzymes of rumen bacteria. Appl Environ Microbiol 43:1026-1033
Kopecny J, Williams AG (1988)
Synergism of rumen microbial hydrolases during degradation of plant polymers. Folia Microbiol 33:208-212
Lamed R, Bayer EA (1988)
The cellulosome of *Clostridium thermocellum*. Adv Appl Microbiol 33:1-46
Lanzani A, Piana G, Piva G, Cardillo M, Rastelli A, Jacini G (1974)
Changes in *Brassica napus* progoitrin induced by sheep rumen fluid. J Am Oil Chem Soc 51:517-518
Latham MJ, Sharpe ME, Sutton JD (1971)
The microbial flora of the rumen of cows fed hay and high cereal rations and its relationship to the rumen fermentation. J Appl Bacteriol 2:425-435
Leat WMF, Harrison FA (1975)
Digestion, absorption and transport of lipids in the sheep. In : McDonald IW, Warner ACI (eds), Digestion and metabolism in the ruminant. University of New England. Armidale, Australia
Leatherwood JM (1973)
Cellulose degradation by *Ruminococcus*. Fed Proc 32:1814-1818
Leedle JAZ, Barsunn K, Butine KJ (1987)
Comparison of treatments for the *in vitro* detachment of cellulolytic bacteria from ruminal digesta samples. Current Microbiol 15:129-131
Lees JA, Oldham JD, Haresign W, Garnsworthy PC (1990)
The effect of patterns of rumen fermentation on the response by dairy cows to dietary protein concentrations. Br J Nutr 63:177-186
Lin KW, Patterson JA, Ladisch MR (1985)
Anaerobic fermentations : microbes from ruminants. Enzyme Microb Technol 7:98-107

Lindell L (1976)
Rapeseed meal in rations for dairy cows. 2. Comparison of two levels of rapeseed meal. Swedish J Agric Res 6:65-71
Lindgren E (1987)
Fodrets Energivärde. Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences. Cited in : Murphy M (1990), Some general characteristics of the ruminal ecosystem in cattle. In : Aspects of rumen microbiology and metabolism. Report 197, Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Uppsala, Sweden .
Lubberding HJ, Gijzen HJ, Gerhardus MJT, Vogels GD (1987)
Fibre degradation and cellulase activities in an artificial rumen system in the presence and absence of rumen ciliates. Proceedings of the IV International Symposium on physiology of ruminant nutrition, High Tatras Štrbské Pleso, Czechoslovakia.
Lubberding HJ, Gijzen HJ, Heck M, Vogels GD (1988)
Anaerobic digestion of onion waste by means of rumen microorganisms. Biol Wastes 25:61-67
McAllister TA, Cheng KJ, Rode LM, Forsberg CW (1990)
Digestion of barley, maize and wheat by selected species of ruminal bacteria. Appl Environ Microbiol 56:3146-3153
Macleod GK, Buchanan-Smith JG (1972)
Digestibility of hydrogenated tallow, saturated fatty acids and soybean oil-supplemented diets by sheep. J Anim Sci 35:890-895
Marangos A, Hill R (1974)
The hydrolysis and absorption of thioglucosides of rapeseed meal. Proc Nutr Soc 33:90A (Abstr.)
McKinley VL, Vestal JR (1985)
Physical and chemical correlates of microbial activity and biomass in composting municipial sewage sludge. Appl Environ Microbiol 50:1395-1403
Minato H, Suto T (1978)
Technique for fractionation of bacteria in rumen microbial ecosystem. II Attachment of bacteria isolated from bovine rumen to cellulose powder *in vitro* and elution of bacteria therefrom. J Gen Appl Microbiol 24:1-16
Moir RJ (1968)
Ruminal digestion and evolution. In : Code CF (ed), Handbook of physiology. Cited in : Hungate RE (1988), The ruminant and the rumen. In : Hobson PN (ed), The rumen microbial ecosystem. Elsevier Applied Science, London-New York
Morris EJ, Cole OJ (1987)
Relationship between cellulolytic activity and adhesion to cellulose in *Ruminococcus albus*. J Gen Microbiol 133:1023-1032
Nugon-Baudon L, Szylit O, Raibaud P (1988)
Production of toxic glucosinolate derivatives from rapeseed meal by intestinal microflora of rat and chicken. J Sci Food Agric 43:299-308
Oginsky EL, Stein AE, Greer MA (1965)
Myrosinase activity in bacteria as demonstrated by the conversion of progoitrin to goitrin. Proc Soc Exp Med 119:360-364
O'Kelly JC, Spiers WG (1990)
Influence of the host diet on the fatty acid composition and content of rumen protozoa in cattle. J Protozool 37:190-193
Orpin CG (1975)
Studies on the rumen flagellate *Neocallimastix frontalis*. J Gen Microbiol 91:249-262
Orpin CG (1976)
Studies on the rumen flagellate *Sphaeromonas communis*. J Gen Microbiol 94:270-280
Orpin CG (1977a)
On the induction of zoosporogenesis in the rumen phycomycetes *Neocallimastix frontalis*, *Piromonas communis* and *Sphaeromonas communis*. J Gen Microbiol 101:181-189
Orpin CG (1977b)
The rumen flagellate *Piromonas communis* : Its life-history and invasion of plant material in the rumen. J Gen Microbiol 99:107-117

Orpin CG (1984)
The role of ciliate protozoa and fungi in the rumen digestion of plant cell walls. Anim Feed Sci Technol 10:121-143
Oshio S, Tahata I, Minato H (1987)
Effect of diets differing in ratios of roughage to concentrate on microflora in the rumen of heifers. J Gen Appl Microbiol 33:99-111
Owens FN (1987)
New techniques for studying digestion and absorption of nutrients by ruminants. Fed Proc 46:283-289
Papas A, Ingalls JR, Cansfield P (1978)
Effect of Tower and 1821 rapeseed meals and tower gums on milk yield, milk composition and blood parameters of lactating dairy cows. Can J Anim Sci 58:671-679
Prins RA (1977)
Biochemical activities of gut microorganisms. In : Clarke RTJ, Bauchop T (eds), Microbial ecology of the gut. Academic Press Ltd, London
Prins RA (1987)
Rumen microbial metabolism of plant secondary compounds, xenobiotics and drugs. In : Ooms LAA, Desgryse AD, van Miert ASJPAM (eds), Physiological and pharmacological aspects of the reticulo-rumen. Martinus Nijhoff Publishers, Dordrecht, The Netherlands
Prins RA (1990)
History and future of rumen microbiology. In : Van Gylswyk N, Lindgren E (eds), Aspects of rumen microbiology and metabolism. Report 197, Swedish University of Agricultural Sciences, Department of Animal Nutrition and Management, Uppsala, Sweden
Prins RA & Clarke RTJ (1980)
Microbial ecology of the rumen. In : Ruckebusch Y, Thivend P (eds), Digestive physiology and metabolism in ruminants. MTP Press Limited, Lancaster, England
Pryde EH, Doty HO Jr (1981)
World fats and oil situation. In : Pryde EH, Princen LH, Mukherjee KD (eds), New sources of fats and oils. American Oil Chemists Society, Champaign, IL, USA
Pusztai A (1989)
Antinutrients in rapeseeds. Nutrition Abstracts and Reviews (Series B) 59:427-433
Rattray JBM (1984)
Biotechnology and the fats and oils industry - an overview. J Am Oil Chem Soc 61:1701-1712
Robson LM, Chambliss GH (1989)
Cellulases of bacterial origin. Enzyme Microb Technol 11:626-644
Rooke JA, Norton BW, Armstrong DG (1982)
The digestion of untreated and formaldehyde-treated soya-bean meals and estimation of their rumen degradability by different methods. J Agric Sci Camb 99:441-452
Rufener WH Jr, Nelson WO, Wolin MJ (1963)
Maintenance of the rumen microbial population in continuous culture. Appl Microbiol 11:196-201
Russell JB, Baldwin RL (1978)
Substrate preferences in rumen bacteria : evidence of catabolite regulatory mechanisms. Appl Environ Microbiol 36:319-329
Russel JB, Wallace RJ (1988)
Energy yielding and consuming reactions. In : Hobson PN (ed),
The rumen microbial ecosystem. Elsevier Applied Science, London-New York
Rutkowski A (1971)
The feed value of rapeseed meal. J Am Oil Chem Soc 48:863-868
Schelling GT (1984)
Monensin mode of action in the rumen. J Anim Sci 58:1518-1527
Sessa DJ, Bietz JA (1986)
Toasted soybean flour components with trypsin inhibitor activity. J Am Oil Chem Soc 63:784-788
Sharma HR, Ingalls JR, McKirdy JA (1977)
Effects of feeding a high level of Tower rapeseed meal in dairy rations on feed intake and milk production. Can J Anim Sci 57:653-662

Shimada K, Cheftel JC (1988)
Determination of sulfhydryl groups and disulfide bonds in heat-induced gels of soy protein isolate. J Agric Food Chem 36:147-153
Shimada K, Matsushita S (1980)
Relationship between thermo-coagulation of proteins and amino acid compositions. J Agric Food Chem 28:413-417
Silva AT, Wallace RJ, Ørskov ER (1987)
Use of particle-bound microbial enzyme activity to predict the rate and extent of fibre degradation in the rumen. Br J Nutr 57:407-415
Slyter LL, Kern DL, Weaver JM, Oltjen RR, Wilson R (1971)
Influence of starch and nitrogen sources on ruminal microorganisms of steers fed high fiber purified diets. J Nutr 101:847-854
Smith RH (1975)
Nitrogen metabolism in the rumen and the composition and nutritive value of nitrogen compounds entering the duodenum. In : Digestion and metabolism in the ruminant : Proceedings of the IV International symposium on ruminant physiology, Sydney, Australia, August 1974. University of New England Publishing Unit, Armidale, Australia.
Sniffen CJ, Chen G, Russell JB (1985)
Concentration and flow of peptides from the rumen of cattle fed different amounts and types of proteins. Report on XVIII Conference on rumen function, Chicago. Cited in : Hobson PN (ed), The rumen microbial ecosystem. Elsevier applied science, London-NY, 1988
Soest PJ van (1973)
The uniformity and nutritive availability of cellulose. Fed Proc 32:1804-1808
Somogyi M (1952)
Notes on sugar determination. J Biol Chem 195:19-23
Sosulski K (1986)
Enzyme use in oil extraction. J Am Oil Chem Soc 63:969
Stewart CS (1977)
Factors affecting the cellulolytic activity of rumen contents. Appl Environ Microbiol 33:497-502
Stewart CS, Bryant MP (1988)
The rumen bacteria. In : Hobson PN (ed), The rumen microbial ecosystem. Elsevier Applied Science, London-New York
Still WC, Kahn M, Mitra A (1978)
Rapid chromatographic technique for preparative separating with moderate resolution. J Org Chem 43:2923-2925
Szakács-Dobozi, Halász A, Kozma-Kovács E, Szakács G (1988)
Enhancement of mustard oil yield by cellulolytic pretreatment. Appl Microbiol Biotechnol 29:39-43
Tapper BA, Reay PF (1973)
Cyanogenic glycosides and glucosinolates. In : Butler CW, Bailey RW (eds), Chemistry and biochemistry of herbage. Academic Press, London-New York
Teunissen MJ, Marras SAE, Op den Camp HJM, Vogels GD (1989)
Improved method for simultaneous determination of alcohols, volatile fatty acids, lactic acid and 2,3 butanediol in biological samples. J Microbiol Meth 10 : 247-254
Teunissen MJ, Op den Camp HJM, Orpin GC, Huis in 't Veld JHJ, Vogels GD (1991a)
Comparison of growth characteristics of anaerobic fungi isolated from ruminant and non-ruminant herbivores during cultivation in a defined medium. J Gen Microbiol 137:1401-1408
Teunissen MJ, Smits AAM, Op den Camp HJM, Huis in 't Veld JHJ, Vogels GD (1991b)
Fermentation of cellulose and production of cellulolytic and xylanolytic enzymes by anaerobic fungi from ruminant and non-ruminant herbivores. Arch Microbiol 156:290-296
Teunissen MJ, Kets E, Op den Camp HJM, Huis in 't Veld JHJ, Vogels GD (1992)
Effect of methanogenic bacteria on cellulolytic and xylanolytic activity of four anaerobic fungi from ruminants and non-ruminants. Arch Microbiol 157
Thangstad OP, Iversen T-H, Slupphaug G, Bones A (1990)
Immunocytochemical localization of myrosinase in *Brassica napus* L. Planta 180:245-248

Thauer RT, Jungermann K, Decker K (1977)
Energy conservation in chemotrophic anaerobic bacteria. Bacteriol Rev 41:100-180
Thomke S (1981)
Review of rapeseed meal in animal nutrition : ruminant animals. J Am Oil Chem Soc 58:805-810
VanEtten CH, Daxenbichler ME, Schroeder W, Princen LH, Perry TW (1977)
Tests for epi-progoitrin, derived nitriles, and goitrin in body tissues from cattle fed crambe meal. Can J Anim Sci 57:75-80
VanEtten CH, Daxenbichler ME, Wolff IA (1969)
Natural glucosinolates (thioglucosides) in foods and feeds. J Agr Food Chem 17:483-491
VanEtten CH, Tookey HL (1978)
Glucosinolates in cruciferous plants. In : Keeler RF, van Kampen KR, James LF (eds), Effects of poisonous plants on livestock. Academic Press, New York
VanEtten CH, Tookey HL (1979)
Chemistry and biological effects of glucosinolates. In : Rosenthal GA, Janzen DH (eds), Herbivores, their interaction with secondary plant metabolites. Academic Press, New York
Van Nevel CJ, Demeyer DI (1988)
Manipulation of rumen fermentation. In : Hobson PN (ed), The rumen microbial ecosystem. Elsevier Applied Science, London-New York
Varel VH, Dehority BA (1989)
Ruminal cellulolytic bacteria and protozoa from bison, cattle-bison hybrids, and cattle fed three alfalfa-corn diets. Appl Environ Microbiol 55:148-153
Varvikko T (1986)
Evaluation of rumen-undegraded feed protein. Thesis, Dept of Animal Husbandry, Agricultural Research Centre, Jokioinen, Finland
Virtanen AI (1961)
Über die Chemie der *Brassica*-Faktoren, ihre Wirkung auf die Funktion der Schildrüse und ihr Übergehen in die Milch. Experientia 17:241-284
Virtanen AI, Kreula M, Kiesvaara M (1958)
The transfer of L-5-vinyl-2-thioxazolidone (oxazolidinethione) to milk. Acta Chem Scand 12:580-581
Virtanen AI, Kreula M, Kiesvaara M (1963)
Investigations on the alleged goitrogenic properties of milk. Z Ernahrungswiss Suppl 3:23-27
Vishniac W, Santer M (1957)
The thiobacilli. Bacteriol Rev 21:195-213
Vos RH de, Blijleven WGH (1988)
The effect of processing on glucosinolates in cruciferous vegetables. Z Lebensm Unters Forsch 187:525-529
Wallace RJ & Cotta MA (1988)
Metabolism of nitrogen-containing compounds. In : Hobson PN (ed), The rumen microbial ecosystem. Elsevier Applied Science, London-New York
Wallace RJ, Joblin KN (1985)
Proteolytic activity of a rumen anaerobic fungus. FEMS Microbiol Lett 29:19-25
Wallace RJ, Munro CA (1986)
Influence of the rumen anaerobic fungus *Neocallimastix frontalis* on the proteolytic activity of a defined mixture of rumen bacteria growing on a solid substrate. Lett Appl Microbiol 3:23-26
Weimer PJ, Lopez-Guisa JM, French AD (1990)
Effect of cellulose fine structure on kinetics of its digestion by mixed ruminal microorganisms *in vitro*. Appl Environ Microbiol 56:2421-2429
Williams AG (1979)
The selectivity of carbohydrate assimilation by the anaerobic rumen ciliate *Dasytricha ruminantium*. J Appl Bacteriol 47:511-520
Williams AG (1986)
Rumen holotrich ciliates. Microbiol Rev 50 :25-49
Williams AG, Coleman GS (1988)
The rumen protozoa. In : Hobson PN (ed), The rumen microbial ecosystem. Elsevier Applied Science, London-New York

Williams AG, Ellis AB, Coleman GS (1986)
Subcellular distribution of polysaccharide depolymerase and glycoside hydrolase enzymes in rumen ciliate protozoa. Curr Microbiol 13:139-147
Williams AG, Orpin CG (1987a)
Polysaccharide-degrading enzymes formed by three species of anaerobic fungi grown on a range of carbohydrate substrates. Can J Microbiol 33:418-426
Williams AG, Orpin CG (1987b)
Glycoside hydrolase enzymes present in the zoospore and vegetative growth stages of the rumen fungi *Neocallimastix patriciarum*, *Piromonas communis* and an unidentified isolate, grown on a range of carbohydrates. Can J Microbiol 33:427-434
Williams AG, Strachan NH (1984)
The distribution of polysacharide-degrading enzymes in the bovine rumen digesta ecosystem. Current Microbiol 10:215-220
Williams AG, Withers SE (1982)
The production of plant cell wall polysaccharide degrading enzymes by hemicellulolytic rumen bacterial isolates grown on a range of carbohydrates. J Appl Bacteriol 52:377-387
Williams AG, Withers SE, Strachan NH (1989)
Postprandial variations in the activity of polysaccharide-degrading enzymes in microbial populations from the digesta solids and liquor fractions of rumen contents. J Appl Bacteriol 66:15-26
Wolin MJ (1975)
Interactions between the bacterial species of the rumen. In : McDonald IW, Warner ACI (eds), Digestion and metabolism in the ruminant. University of New England Publishing Unit, Armidale, Australia
Wolin MJ (1979)
The rumen fermentation : a model for microbial interaction in anaerobic ecosystems. In : Alexander M (ed), Advances in microbial ecology, vol 3. Plenum, New York
Wolin MJ (1981)
Fermentation in the rumen and human large intestine. Science 213:1463-1468
Wolin MJ, Miller TL (1983)
Interactions of microbial populations in cellulose fermentation. Federation Proc 42:109-113
Wolin MJ, Miller TL (1988)
Microbe-microbe interactions. In : Hobson PN (ed), The rumen microbial ecosystem. Elsevier Applied Science, London-New York
Wong KKY, Tan LUL, Saddler JN (1988)
Multiplicity of β-1,4-xylanase in microorganisms : functions and applications. Microbiol Rev 52:305-317
Wood TM, McCrae SI (1977)
Cellulase from *Fusarium solani*. Purification and properties of the C_1 component. Carbohydr Res 57:117-133
Wood TM, Wilson CA, McCrae SI, Joblin KN (1986)
A highly active extracellular cellulase from the anaerobic fungus *Neocallimastix frontalis*. FEMS Microbiol Lett 34 :37-40

SUMMARY / SAMENVATTING

SUMMARY

The rumen of herbivores such as dairy cows and sheep contains a complex mixture of bacteria, protozoa and fungi. These microorganisms extensively degrade dietary material prior to enzymatic gastric digestion. This symbiotic relationship between ruminant and microorganisms enables the animal to convert huge amounts of plant polymers that cannot be degraded by animal digestive processes. Overall about 70 to 80 % of the total dietary organic matter is degraded in the rumen. In the rumen of a typical dairy cow, for instance, 60 kg plant carbohydrates can be fermented daily.

Plant constituents are converted to volatile fatty acids (mainly acetic, propionic and butyric acid), CO_2, CH_4, ammonia and microbial cells. The volatile fatty acids formed serve as a major energy source for the ruminant, whereas the microbial cells are major protein and vitamin sources.

This thesis evaluates the potency of an artificial rumen system for applications in food industry. This *in vitro* rumen system, developed recently for the degradation of organic wastes, closely resembles the *in vivo* system with respect to fermentation products, degradation efficiencies, numbers of ciliated protozoa and the difference in solid and liquid retention times.

Chapter 1 describes rumen functions, the normal microbial population and quantitative aspects of rumen fermentation. Soybeans and rapeseed are both major sources of edible fats and oils. The meal remaining after the extraction of the oil is used as a protein feed for livestock. However, the use of these meals is limited by the presence of antinutrional compounds. These plant raw materials, used in most tests described in this thesis, are introduced in this chapter.

General methods, predominantly concerning the set-up of the artificial rumen system, are described in Chapter 2.

The effect of soybean or rapeseed meal supplementation to ruminal diets is usually tested *in vivo*. Comparison of the results is hindered by the use of different diets and animals, and consequently differing rumen parameters. In an *in vitro* system these supplementations can be tested under standardized conditions, as the sole substrate for the system and at high loading rates without the danger of toxicity or problems with palatability. The use of the artificial rumen system to predict degradation efficiencies is studied in Chapter 3. Comparison of the results of the *in vitro* system with the results of feeding trials *in vivo* showed that the artificial rumen system is a good model to study the rumen digestibility of plant raw materials used as feed ingredients. Effects of various loading regimes and physico-chemical parameters and the presence of microorganisms in the system can easily be tested in this *in vitro* system.

Lignocellulosic materials are degraded by (hemi)cellulolytic enzymes present in or excreted by rumen microorganisms. In Chapter 4 the (hemi)cellulolytic activities in cell-free extracts from the artificial rumen system are compared with the activities calculated on base of degradation efficiencies in the intact system. The enzyme levels in these extracts were about ten times lower than the levels calculated from the performance of the intact system. It appeared that the enzymes are not easily detached from the microorganisms or the substrates to which they are bound *in vivo*. Furthermore, a

considerable amount of the activity is lost as a consequence of the extraction treatment applied. This is probably due to a loss of the cellular or molecular organization. The strong binding and the loss of activity of the (hemi)cellulolytic enzymes of rumen microorganisms hampers the use of these enzymes for biotechnological purposes.

In order to investigate the specific induction of (hemi)cellulolytic enzymes in the artificial rumen system, different substrates were applied as feed in Chapter 5. Filter paper cellulose, low protein soybean meal and low protein rapeseed meal were tested. Although these substrates differed markedly in composition, the specific (hemi)cellulolytic activities in cell-free extracts from these systems appeared to be rather similar. Induction, by variation of the diet, of enzymes especially suited to degrade soybean or rapeseed cell walls, which could be used to enhance oil extraction from rapeseed and soybeans, did not seem to occur.

The extraction of oil from oilseeds, as soybeans and rapeseed, can be facilitated by the use of enzymes. These enzymes degrade the cell walls of the seeds before oil extraction. In Chapter 6 the potential use of artificial rumen fractions in the extraction of oil from oilseeds was tested. The application of complete, untreated fractions of the artificial rumen system resulted in high oil yields (up to 60 % of the total amount of extractable oil) in contrast to cell-free extracts, which disclosed up to 21 % of the total amount of extractable oil. The use of intact rumen microorganisms for this purpose is limited because they are not Generally Recognized As Safe (GRAS). In addition a commercial enzyme preparation and the extracellular (hemi)cellulolytic enzymes produced by pure cultures of anaerobic fungi, microorganisms actively involved in fibre degradation in the rumen, were also tested. These experiments revealed that the extracellular enzymes from anaerobic fungi might be better suited for the improvement of oil extraction than cell-free extracts of the complex microbial population of the rumen.

The protein rich meals, co-products of the oil extraction from soybeans and rapeseed contain antinutritional compounds. In soybean meal protease inhibitors and in rapeseed meal glucosinolates, tannins and erucic acids are the major groups of antinutritional factors. Ruminants are usually less susceptible for the presence of these antinutritional compounds in their feed than monogastric animals like pigs and poultry. The effect of the antinutritional factors is generally tested *in vivo*. A diet containing these antinutritional compounds is fed, and animal products, like milk, meat and sometimes faeces and urine, are tested for the presence of antinutritional compounds or their derivatives. The artificial rumen system was used to test the effect of toxic products from glucosinolates, antinutritional factors present in rapeseed, on rumen performance. These experiments are described in Chapter 7. One of the products formed from progoitrin (2-hydroxy-3-butenyl glucosinolate), an antinutritional compound normally present in rapeseed is L-5-vinyloxazolidine-2-thione (VOT). The VOT was completely degraded in the rumen when concentrations up to 4 mM were added continuously with the influent buffer. The VOT degrading capacity of the artificial rumen reactor amounts to at least 8 mmol per litre per day, an amount equivalent to approximately 160 g rapeseed meal per litre per day, four times the normal loading rate of the system. This may explain the observed resistance of ruminants to the inclusion of rapeseed meal in their diet.

In conclusion, the artificial rumen system appeared to be an excellent tool to test the effect of specific dietary ingredients on rumen performance under standardized conditions *in vitro* and to study metabolic processes which occur in the rumen. For the production and application of (hemi)cellulolytic enzymes, however, specific groups of rumen microorganisms, like anaerobic fungi, are better suited.

SAMENVATTING

De pens van herkauwers, zoals koeien en schapen, bevat een complex mengsel micro-organismen : bacteriën, protozoën en schimmels. Deze micro-organismen fermenteren het voedsel, waarna de enzymatische omzettingen plaatsvinden in de echte maag. De symbiotische relatie tussen herkauwer en micro-organismen stelt het dier in staat grote hoeveelheden plantaardig materiaal om te zetten, die niet kunnen worden afgebroken door de verteringsprocessen van het dier zelf. Globaal kan ongeveer 70 tot 80 % van het organisch materiaal in het dieet worden afgebroken in de pens. In de pens van een typische melkkoe, bijvoorbeeld, kunnen dagelijks 60 kg plantaardige koolhydraten worden afgebroken.

Plantaardig materiaal wordt omgezet in vluchtige vetzuren (voornamelijk azijn-, propion- en boterzuur), kooldioxide, methaan, ammonia en microbiële cellen. De gevormde microbiële cellen vormen een belangrijke energiebron voor de herkauwer. Daarnaast zijn deze cellen ook van belang als bron van eiwitten en vitamines.

Dit proefschrift evalueert de toepassingsmogelijkheden van een kunstpenssysteem in de voedingsmiddelenindustrie. Dit *in vitro* penssysteem, dat enkele jaren geleden werd ontwikkeld voor de afbraak van organisch afval, lijkt sterk op het *in vivo* systeem wat betreft fermentatieprodukten, afbraakefficiënties, ciliatenaantallen en het verschil in vloeistof en vaste stof verblijftijden.

Hoofdstuk 1 beschrijft de functies van de pens, de microbiële populatie en enkele kwantitatieve aspecten van de pensfermentatie. Daarnaast worden de plantaardig materialen, die in bijna alle, in dit proefschrift beschreven, experimenten worden gebruikt, in dit hoofdstuk geïntroduceerd, t.w. sojabonen en raapzaad. Deze zaden zijn belangrijke bronnen van eetbare oliën en vetten. Het schroot, dat na extractie van de olie overblijft wordt gebruikt als een eiwitbron in veevoeders. Het gebruik van dit schroot in veevoeders wordt echter beperkt door de aanwezigheid van antinutritionele factoren.

Algemene methoden, voornamelijk wat betreft het opzetten van het kunstpenssysteem, worden beschreven in Hoofdstuk 2.

Het effect van het toevoegen van soja- of raapzaadschroot aan het dieet voor de herkauwer wordt meestal *in vivo* getest. Vergelijking van de resultaten van verschillende experimenten wordt bemoeilijkt door het feit dat verschillende dieten en dieren en dientengevolge verschillende pensparameters gebruikt worden. In een *in vitro* systeem kunnen deze toevoegingen onder gestandaardiseerde condities getest worden, als het enige substraat voor het systeem en met hoge belastingen zonder gevaar voor vergiftiging van het dier of problemen met de eetlust. Het gebruik van het kunstpenssysteem om afbraakefficiënties te voorspellen wordt beschreven in Hoofdstuk 3. Vergelijking van de resultaten van het *in vitro* systeem met de resultaten van dierproeven hebben laten zien dat het kunstpenssysteem gebruikt kan worden om de afbraakefficiëntie van veevoederingrediënten in de pens te testen. Het effect van vele belasting-regimes op fysisch-chemische parameters en op de aanwezigheid van micro-organismen in het systeem kan gemakkelijk bepaald worden in dit *in vitro* systeem.

Lignocellulose wordt afgebroken door (hemi)cellulolytische enzymen aanwezig in de celwand van of uitgescheiden door pensmicro-organismen. In Hoofdstuk 4 wordt de (hemi)cellulolytische activiteit in cel-vrije extracten uit het kunstpenssysteem vergeleken met de activiteit berekend op basis van de afbraakefficiëntie van filtreerpapier in het intacte systeem. De enzymatische activiteit in deze extracten was ongeveer tien maal zo laag als de activiteit berekend op basis van het functioneren van het intacte systeem. De (hemi)cellulolytische enzymen worden waarschijnlijk niet makkelijk losgemaakt van de micro-organismen en de substraten waaraan zij in de pens gebonden zijn. Daarnaast gaat een behoorlijk deel van de activiteit verloren als een effect van de gevolgde extractie procedure. Dit is waarschijnlijk een gevolg van het verlies van de cellulaire of moleculaire organisatiestructuur. De sterke binding en het activiteitsverlies van de (hemi)cellulolytische enzymen van pensmicro-organismen beperkt het gebruik van deze enzymen voor biotechnologische doeleinden.

Om de specifieke inductie van (hemi)cellulolytische enzymen in het kunstpens-systeem te bestuderen werden verschillende substraten als voedingsbron voor het systeem gebruikt (Hoofdstuk 5). Filtreer papier (100 % cellulose), eiwitarm sojaschroot en eiwitarm raapzaadschroot werden getest. Hoewel deze substraten duidelijk verschillen in samenstelling, bleken de specifieke (hemi)cellulolytische activiteiten in celvrije extracten van deze systemen sterk op elkaar te lijken. Het bleek niet mogelijk om door variatie van het dieet specifieke enzymen te induceren om soja- en raapzaad-celwandmaterialen af te breken en te gebruiken in olie-extractie processen.

De extractie van olie uit oliehoudende zaden, zoals soja en raapzaad, kan worden bevorderd door het gebruik van enzymen. Deze enzymen breken de celwanden van deze zaden af voor de olie-extractie. In Hoofdstuk 6 wordt het mogelijk gebruik van fracties van het kunstpenssysteem in de extractie van oliehoudende zaden getest. Het gebruik van intacte, onbehandelde fracties uit het kunstpenssysteem resulteerde in hoge olie-opbrengsten (tot 60 % van de totale hoeveelheid extraheerbare olie) in tegenstelling tot het gebruik van celvrije extracten, die ten hoogste 21 % van de totale hoeveelheid extraheerbare olie ontsloten. Het gebruik van intacte pensmicro-organismen voor dit doel is beperkt omdat deze niet "Generally Recognized As Safe" (GRAS) zijn. Daarnaast zijn een commercieel enzym preparaat en de extracellulaire (hemi) cellulolytische enzymen geproduceerd door reincultures van anaerobe schimmels, micro-organismen betrokken bij de vezelafbraak in de pens, getest. Op grond van deze experimenten kan geconcludeerd worden dat de extracellulaire enzymen van anaerobe schimmels waarschijnlijk beter geschikt zijn om de olie-extractie te bevorderen dan celvrije extracten van de complexe microbiële populatie in de pens.

Het eiwitrijke schroot, bijprodukt van de olie-extractie uit soja en raapzaad, bevat antinutritionele factoren. In sojaschroot vormen proteaseremmers en in raapzaadschroot glucosinolaten, tannines and erucinezuur de belangrijkste groepen antinutritionele factoren. Herkauwers zijn over het algemeen minder gevoelig voor deze antinutritionele factoren in hun voedsel dan eenmagigen zoals kippen en varkens. Het effect van de antinutritionele componenten wordt meestal *in vivo* getest. Er wordt een dieet gevoerd dat deze antinutritionele factoren bevat en dierlijke produkten, zoals melk, vlees en soms faecaliën en urine, worden getest op de aanwezigheid van de gevoerde antinutritionele componenten of produkten daarvan. Het kunstpenssysteem werd

gebruikt om het effect van toxische produkten van glucosinolaten, antinutritionele factoren uit raapzaad, op de pensfermentatie te testen. Deze experimenten worden beschreven in Hoofdstuk 7. Een van de produkten gevormd uit progoitrine (2-hydroxy-3-butenylglucosinolaat), een antinutritionele factor uit raapzaad, is L-5-vinyloxazolidine-2-thion (VOT). Dit VOT werd volledig afgebroken in de pens, wanneer het werd toegevoegd in concentraties tot 4 mM in de instroombuffer. De afbraakcapaciteit voor VOT van het kunstpenssysteem bedraagt minstens 8 mmol per liter per dag, een hoeveelheid die overeenkomt met ongeveer 160 g raapzaadschroot per liter per dag, vier maal zoveel als normaal gevoerd wordt. Dit is een mogelijke verklaring voor de resistentie van herkauwers tegen het voeren met raapzaadschroot.

Concluderend kan opgemerkt worden dat het kunstpenssysteem zeer geschikt is om *in vitro* het effect van specifieke onderdelen van het dieet op het functioneren van het penssysteem onder gestandaardiseerde condities te testen en het metabolische effect van deze componenten te bestuderen. Specifieke groepen pensmicro-organismen, zoals anaerobe schimmels, zijn waarschijnlijk beter geschikt voor de produktie en de toepassing van (hemi)cellulolytische enzymen.

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CURRICULUM VITAE

Elisabeth Johanna Maria Geertman werd op 3 augustus 1960 geboren te Nijmegen. In 1978 behaalde zij het VWO-diploma aan het Canisius College - Mater Dei te Nijmegen. In datzelfde jaar begon zij met de studie biologie aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen werd in november 1981 afgelegd. Het doctoraalprogramma omvatte als hoofdvak microbiologie (prof. dr. ir. G.D. Vogels) en als bijvakken aquatische ecologie (prof. dr. C. den Hartog) en preventieve en sociale tandheelkunde (dr. J.S. van der Hoeven). Daarnaast werd een onderwijsbevoegdheid behaald. Het doctoraalexamen werd in november 1985 afgelegd.

Van februari 1986 tot januari 1991 was zij als wetenschappelijk medewerkster werkzaam op het Laboratorium voor Microbiologie van de Katholieke Universiteit te Nijmegen in het kader van een door de Hoofdgroep TNO - Voeding gefinancierd onderzoek.

Sinds 22 augustus 1991 is zij als docent verbonden aan de Hogeschool Eindhoven.