Metabolism of Soluble Proteins by Rumen Microorganisms and the Influence of Condensed Tannins on Nitrogen Solubility and Degradation

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Abstract

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The amino acid requirements of ruminants are met by two sources; microbes leaving the rumen and dietary protein escaping fermentation in the rumen. Much ruminant research has therefore focused on improving amino acid supply to the duodenum by increasing both microbial protein synthesis and escape of feed proteins from the rumen. The escape of dietary protein is dependent on the degradation characteristics and retention time in the rumen.

The overall aims of this thesis were to increase the knowledge of ruminal degradation of buffer soluble proteins and to investigate the effect of condensed tannins on the degradation. The thesis examines the effect of trichloroacetic acid, perchloric acid, and tungstic acid on detection and recovery of feed peptides and chemically-defined peptides. Twenty-five feeds were screened for buffer soluble protein N. *In vitro* ruminal degradation rates of buffer soluble proteins were estimated in 11 of these feeds. Buffer soluble protein from peas or cold-pressed rapeseed cake was given as a pulse dose together with a liquid marker to lactating dairy cows, to investigate rumen *in vivo* degradation rates. The concentration of condensed tannins was determined for birdsfoot trefoil (*Lotus corniculatus* L.), big trefoil (*L. uliginosus* Schkur.) and sainfoin (*Onobrychis viciifolia* Scop.) and related to nitrogen solubility in fresh-frozen and ensiled material and ruminal *in vitro* degradation.

The different protein precipitants did not alter detection of peptides formed in a ruminal *in vitro* system. The recovery of an eight-residue peptide was 0.66, 0.88, and 0.91 for tungstic acid, perchloric acid, and trichloroacetic acid, respectively. The content of soluble protein N in feeds ranged from 0 to 874 g/kg buffer soluble N with the highest contents for lupine, peas, and cold-pressed rapeseed cake. Fractional degradation rates determined *in vitro* for soluble protein ranged from 0.18 (linseed cake) to 1.0 h^{-1} (casein). Soluble protein from soybean meal, peas, and lupine were degraded at intermediate rates. Soluble proteins given as a pulse dose were rapidly degraded *in vivo*. Results were disturbed by slow mixing in the rumen. In several cases, concentration of the liquid marker was higher after 1 h than 0.5 h post dosing.

Based on two experiments and a total of nine varieties of birdsfoot trefoil, the concentration of condensed tannins determined by a radial diffusion method ranged from 3 to 17 g/kg DM. The tannin content for sainfoin and big trefoil was 21.6 (maximum concentration) and 24.8 g/kg DM, respectively. Buffer N solubility and ruminal *in vitro* N degradability were negatively correlated to tannin content. Tannin content and wilting time cumulatively reduced buffer soluble nitrogen in birdsfoot trefoil silage. This thesis shows that there is no distinct cut-off for peptide size when using different protein precipitation agents. Soluble proteins degrade at different rates depending on source. As they are not instantly degraded, they must contribute to the dietary amino acid supply of the ruminant. The *in sacco* method, which assumes complete degradation of soluble proteins should not be used for feeds high in soluble proteins.

Keywords: Soluble proteins, peptides, degradation, *in vitro, in vivo*, tannins, birdsfoot trefoil, sainfoin

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From the moment I picked up your book until I laid it down, I was convulsed with laughter. Some day I intend reading it.

Groucho Marx

Contents

Appendix

Papers I–IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I Hedqvist, H., Broderick, G. & Udén, P. 2004. Recovery of N in amino acids in an automated ninhydrin assay and effect of protein precipitant on peptide recovery. *Submitted.*

II Hedqvist, H. & Udén, P. 2004. Measurement of soluble protein degradation in the rumen. *Submitted*.

III Hedqvist, H., Mueller-Harvey, I., Reed, J.D., Krueger, C.G. & Murphy, M. 2000. Characterisation of tannins and *in vitro* protein digestibility of several *Lotus corniculatus* varieties. Animal Feed Science and Technology 87, 41-56.

IV Hedqvist, H. & Udén, P. 2004. Effect of tannin content in two *Lotus* species and *Onobrychis viciifolia*, with and without the addition of polyethylene glycol, on buffer soluble N. *Manuscript.*

The reprint of Paper **III** is published by kind permission of the journal concerned.

List of abbreviations

AA	Amino acids
AAT	Amino acids absorbable in the small intestine
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
BSN	Buffer soluble nitrogen
CRF	Clarified rumen fluid
CT	Condensed tannins
DM	Dry matter
DW	Distilled water
EPD	Effective protein degradation
GPC	Gel permeation chromatography
HPLC	High performance liquid chromatography
IIV	Inhibitor *in vitro*
MCD	McDougall's buffer
MALDI-TOF	matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry
N	Nitrogen
NAN	Non ammonia nitrogen
NPN	Non protein nitrogen
PA	Proanthocyanidin
PCA	Perchloric acid
PEG	Polyethylene glycol
PRP	Proline-rich proteins
SRF	Strained rumen fluid
TA	Tungstic acid
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
VFA	Volatile fatty acids

Introduction

The protein metabolism in ruminants is a fascinating and complicated story. Ruminants live in symbiosis with microorganisms and this is where the story begins. The host animal supplies the microorganisms in the rumen with plant material, which often is of low nutritional quality, and in return gets access to high-quality microbial protein from microbes. The Finnish chemist Virtanen (1966) showed that dairy cows could produce over 4000 kg milk per year, when fed a diet solely consisting of urea (nitrogen source), cellulose, starch and sugar (carbohydrate sources), vegetable oil, minerals, and fat soluble vitamins.

Ruminants have a more complex protein metabolism than non-ruminants. In monogastrics the amino acid supply for absorption in the small intestine depends on the amount and composition of the feed proteins, whereas in ruminants, the amino acid supply comes from two sources: feed and microorganisms.

Rumen microorganisms, mainly bacteria, degrade feed proteins to peptides, amino acids, and ammonia. Different bacteria use these metabolites to synthesize microbial proteins. Microorganisms are continuously passing out of the rumen with the digesta. When microbes and digesta reach the abomasum, they are subjected to enzymatic and chemical protein degradation, similar to that in the stomach of monogastrics. The proteins of microbial origin constitute an important part of the protein that is available for absorption in the small intestine of ruminants. If there is a lack of energy for the microbes, a considerable amount of the protein, degraded to ammonia, will not be used for microbial protein synthesis. Ammonia is absorbed through the rumen wall and transported to the liver where it is converted to urea. The urea can both recycle to the rumen, mainly via the saliva, or be excreted in the urine.

Most soluble plant proteins are rapidly degraded in the rumen (Mangan, 1972; Volden, Mydland & Olaisen, 2002). A rapid degradation of proteins, combined with energy deficiency, leads to high rumen ammonia concentrations and increased urinary N losses. The N efficiency of grass- and legume-based diets for milk production is typically around 20% (Tamminga, 1992; Bertilsson & Murphy, 2003). If part of the herbage protein can escape ruminal degradation and be available for post ruminal absorption, this can lead to increased protein efficiency.

Some fodder legumes, like birdsfoot trefoil and sainfoin, contain condensed tannins. Condensed tannins may improve the protein utilization by forming rumen-stable complexes with feed proteins, thereby protecting part of the protein from ruminal degradation (Douglas *et al*., 1995). The stability of these complexes is pH-dependent (Jones & Mangan, 1977) and they may dissociate when the pH drops in the abomasum, enabling digestion and subsequent absorption of the free amino acids from the duodenum.

For a low-producing animal such as a dry cow, the supply of microbial protein is sufficient to meet the protein requirements (Van Soest, 1994). A high-producing dairy cow, yielding 40–50 kg milk per day and a total milk protein yield of around 1.5 kg per day, has a protein requirement that cannot be matched by the supply of

microbial protein. These animals must be supplemented with feed protein of high quality that also can escape microbial degradation in the rumen.

Several protein evaluation systems have been developed to describe and predict the amino acid uptake in dairy cows. Examples of these are: the North American NRC system (NRC, 2001), the Dutch DVE/OEB system (Tamminga *et al.*, 1994) and the Nordic AAT/PBV system (Madsen *et al.*, 1995). Although there are differences among the systems, they all try to estimate the contribution of feed and microbes to the amino acid supply of the animal. They also rely on the *in sacco* technique for estimating protein degradation in the rumen. Briefly, feed samples are incubated in nylon bags in the rumen of rumen-fistulated cows for different time intervals. The pore size of the bags is sufficiently large to allow microorganisms to enter the bags and for fermentation end-products not to accumulate, while at the same time small enough to retain particulate matter. The assumption is that all feed material that is removed by only washing the bags without any rumen incubation is instantly degraded. Documented weaknesses with the *in sacco* procedure are feed particle loss, microbial contamination, and low reproducibility (see Hvelplund & Weisbjerg, 2000). Furthermore, the i*n sacco* method cannot be used to estimate degradation of any soluble feed fraction as solubility (disappearance from the bags) must be assumed to be equivalent to degradability. Consequently, soluble protein degradation should be studied using alternative techniques, *e.g. in vitro* or *in vivo* methods.

Proteins: function, structure and solubility

The name protein derives from the Greek *proteios*, which means primary. The name was well chosen, because proteins are involved in almost everything that occurs in the living cell. There are thousands of different proteins in a typical cell, each having a specific function. Proteins can be classified in different ways, *e.g.* by function, solubility or whether they are simple or conjugated. Simple proteins will only yield amino acids or its derivatives upon hydrolysis, while conjugated proteins, in addition to amino acids, also will yield a prosthetic group, *e.g.* the haemegroup in the protein haemoglobin. Based on function, proteins can for example be divided into enzymes, nutrient and storage proteins, transport proteins, structural proteins, regulatory proteins (*e.g.* hormones) and defence proteins (antibodies).

Amino acids, the building blocks of proteins, are characterized by having a basic amino group ($–NH_2$) and an acidic carboxylic group (–COOH). Most amino acids in proteins are α-amino acids, *i.e.* the amino group is attached to a carbon atom adjacent to the carboxyl group. Proline is usually counted as an α-amino acid, but by strict definition it is an α-imino acid because it contains an imino group (–NH) instead of an amino group. Amino acids are linked by peptide bonds. The formation of one peptide bond results in the loss of one amino group and one carboxyl group. For peptides containing between two (dipeptide) and twenty amino acid residues (eicosapeptide), the term oligopeptide is used. Longer polymers are termed polypeptides or proteins. There is no distinct border between polypeptides and proteins, even though 50 amino acid residues are sometimes

used. Thus, the hormone insulin, having 51 α–amino acids has been referred to both as a small protein and a large polypeptide (Barrett & Elmore, 1998).

The structure of proteins can be considered at four levels. The sequence of amino acids in a protein is known as the primary structure. The sequence is genetically coded and is responsible for the shape and physical properties of the protein and thereby also its function. The secondary structure refers to the regular, recurring arrangements of adjacent amino acid residues in a polypeptide chain. The most common secondary structures are the α-helix and the β-conformation. The tertiary structure refers to the spatial relationship between all amino acids in a polypeptide, *i.e*. the α-helix is a part of the tertiary structure. Proteins with more than one polypeptide chain have one more level of structure; the quaternary structure, which refers to the spatial relationship of the polypeptides (subunits) within the protein.

In biochemistry, proteins have traditionally been classified according to their solubility in different solvents. Albumins are soluble in water, globulins in salt solutions, prolamines in aqueous alcohol, and glutelins in dilute alkali (see Blethen *et al*., 1990). Most seed proteins contain some of the fractions. Cereals, like maize, wheat, and rice, contain large amounts of prolamines and glutelines. Legumes contain mainly albumins and globulins (Van Soest, 1994)

Characterisation and analyses of feed N

Generally, animal feedstuffs are analysed for total N and by multiplying the crude protein (CP) content reported by the factor 6.25. This approach assumes that; 1) all N in the feed derives from proteins, and 2) the N content in all proteins is 160 g/kg protein. Regarding the first assumption, there are large differences between feeds. In silage, due to extensive proteolysis, more than half of the N can be non-protein nitrogen (NPN) in the form of peptides, free amino acids, amines, ammonia, and nitrate. Regarding the second assumption, the N content in the 20 standard amino acids of proteins ranges from 80 (tyrosine) to 270 (arginine). Thus, depending on the amino acid composition, every protein has its individual N content. Milk protein uses a factor of 6.38 and for wheat protein it is 5.98.

Feed N can be fractionated according to the buffer and detergent solubility characteristics. The Cornell Net Carbohydrate and Protein System (CNCPS; Sniffen *et al.*, 1992) uses both a rumen-like buffer and neutral and acid detergents as well as protein precipitants to classify feed N into nutritionally significant fractions. Van Soest (1994) pointed out that merely classifying proteins by their solubility is insufficient as it ignores the presence of true proteins and NPN (peptides, amino acids, ammonia and other end-products from fermentation). In the CNCPS system (Sniffen *et al.*, 1992), acid detergent insoluble N is indigestible and NPN is solely used for microbial growth. The intermediate true protein fractions degrade from rapidly (buffer soluble proteins) to slowly (neutral detergent insoluble proteins) according to the system. Even with a relatively simple analysis such as the buffer solubility assay, there is a considerable variation in reported N solubility, both among and within feeds and among buffers as seen in Table 1. There are also large discrepancies among reports using the same buffer

and apparently similar feeds with oats ranging from 133 to 790 g kg^{-1} N when extracted with autoclaved rumen fluid.

Table 1. *Solubility of N in four feeds by various solvents (g/kg total N).*

Feed	Solvent[a]						
Sources[b]	BMM	NaCl	MCD	BP	BCP	DDW	ARF
Oats							
A	268	92					133
B	368	175	489				185
C	413	398	662	554	520		790
D	351	217[c]				115	
E		67	155				
F	258						
Wheat							
B	217	256	292				208
D	436	614[c]				462	
E		403	406				
F	297						
G			370				
Soybean meal							
A	197	130					63
C	193	215	208	140	152		113
D	241	279[c]				198	
F	130						
G			169				
Timothy hay							
A	295	276					222
C	245	236	274	274	320		236
G			354				

[a]*BMM, Burroughs mineral mixture diluted to 10% with distilled water; NaCl, sodium chloride solution (0.5-0.9%); MCD, McDougall's artificial saliva; BP, borate-phosphate buffer; BCP, bicarbonate-phosphate buffer; DDW, distilled deionised water; ARF, autoclaved rumen fluid.*
[b]*(A) Crawford et al., 1978 ; (B) Crooker et al., 1978; (C) Krishnamoorthy et al., 1982; (D) Blethen et al., 1990; (E) Kandylis & Nikokyris, 1997; (F) Wohlt, Sniffen & Hoover, 1973; (G) paper II.*
[c]*Sum of consecutive extraction in DDW and NaCl.*

Protein metabolism by microorganisms

Protein constitutes the major part of feed N in most ruminant diets. In the rumen, ingested proteins are largely degraded to ammonia and volatile fatty acids (VFA). The process includes protein hydrolysis, peptide degradation, amino acid deamination, and fermentation of carbon skeletons (Cotta & Hespell, 1986a). Proteases are mainly associated with the cell surface of bacteria (Kopecny & Wallace, 1982). Thus, the first step in protein degradation is adsorption of soluble

proteins to the bacterial surface (Nugent & Mangan, 1981; Wallace, 1985) or attachment of bacteria, in the case of insoluble proteins (Broderick, Wallace & Ørskov, 1991).

Generally all of the enzymes that convert protein to ammonia in the rumen are assumed to be of microbial origin (Broderick, Wallace & Ørskov, 1991; NRC, 2001). Zhu *et al.* (1999) challenged this assumption, by suggesting that plant enzymes could be partly responsible for herbage protein degradation in grazing animals. Wallace *et al.* (2001) investigated the plant protease theory by adding fresh or autoclaved ryegrass to rumen fluid *in vitro* and found a higher ammonia production for fresh than for autoclaved grass. Many strains and species of bacteria, protozoa, and anaerobic fungi have been found to be proteolytic and they contain a variety of proteolytic enzymes. (Wallace, 1996). Up to 43% of the bacteria isolated from rumen fluid showed proteolytic activity (Prins *et al.*, 1983). Probably the most numerous proteolytic bacterium is *Prevotella ruminicola* (Wallace, Onodera & Cotta, 1997) and in silage-based diets it can constitute more than 60% of the bacteria in the rumen (Van Gylswyk, 1990). The main role of ciliate protozoa in protein degradation is probably hydrolysis of particulate protein of appropriate size, *e.g.* chloroplasts and predation of bacteria. Although studies of fungi are somewhat conflicting, these probably play a minor role in protein degradation (Wallace, Onodera & Cotta, 1997). Hydrolysis of proteins releases oligopeptides, which are broken down to smaller peptides and finally to amino acids (Wallace, Onodera & Cotta, 1997). Ammonia, amino acids and peptides can be taken up by rumen microorganisms and used for synthetic purposes. Ammonia is the predominant source of N for protein synthesis by rumen microorganisms and most rumen bacteria can use ammonia (Wallace, Onodera & Cotta, 1997). In some species, peptides are preferred over amino acids and CNCPS assumes a specific requirement of peptides for amylolytic bacteria (Sniffen *et al.*, 1992).

Tannins

Originally the term tannin was applied to phenol-rich plant extracts that were used to tan hides and convert them into leather. By crosslinking collagen proteins in the hides, the tannins made the leather durable and resistant to microorganisms (Mangan, 1988). Increased knowledge about tannins has contributed to different definitions of tannins throughout the years. These definitions have tended to be either too narrow or too broad (see Zucker, 1983). Tannin is usually applied to the heterogenic group of water-soluble polyphenols, which have the ability to bind to proteins and other macromolecules, like carbohydrates, nucleic acids and steroids. The molecular weights for these compounds are usually in the range of 500 to 3000 Da (Mangan, 1988; Zhu *et al.*, 1997). Very small compounds will not have enough hydroxyl-groups to be reactive and very large compounds will be immobile. They are nutritionally interesting as they can reduce protein degradation in the rumen. Tannins are usually divided into two subgroups; hydrolysable tannins and condensed tannins.

Hydrolysable tannins

Hydrolysable tannins are divided into two types; the gallotannins and the ellagitannins. Both types consist of a polyol core, usually glucose, where the hydroxyl groups are esterified with gallic or galloylgallic acid (gallotannins) or the gallic acid dimer hexahydroxydiphenic acid (ellagitannins) (Lowry *et al*., 1996). As the name suggests, these tannins are easily hydrolysed, either under acidic or basic conditions or by enzymes (Hagerman & Butler, 1991). In contrast to condensed tannins, hydrolysable tannins have a rather limited distribution in nature. They occur in leaves, fruits, pods, wood, and bark in some tree species, *e.g*. oak and chestnut (Lewis & Yamamato, 1989), and they are known to be toxic to ruminants (see Reed, 1995).

Condensed tannins

Condensed tannins are oligomers and polymers of flavanoid units (Hagerman & Butler, 1991). The precursor monomers are flavan-3-ols, where position 3 (see Fig 1) on the C-ring, is hydroxylated. Carbon-carbon bonds link these monomers. In contrast to hydrolysable tannins, the bonds are more resistant to hydrolysis (Hagerman & Butler, 1991). The classification of condensed tannins depends on: 1) type of binding between the monomers, *i.e.* C4→C6 or C4→C8; 3) the different stereoisomers possible, where carbon atom 2, 3, and 4 can participate; 4) the number of hydroxyl groups on the A- and B-rings (Zucker, 1983).

Upon heating and in the presence of acid, the interflavan bonds are cleaved by oxidation and anthocyanidins are formed (Porter *et al.*, 1986). Thus, condensed tannins are also called proanthocyanidins (PA). The most common type of PA is procyanidin (Fig. 1), which has two hydroxyl groups on the B-ring, followed by prodelphinidin with OH-groups on carbon 3', 4' and 5' of the B-ring. Propelargonidin has one OH-group on the B-ring and is rarer (Mangan, 1988).

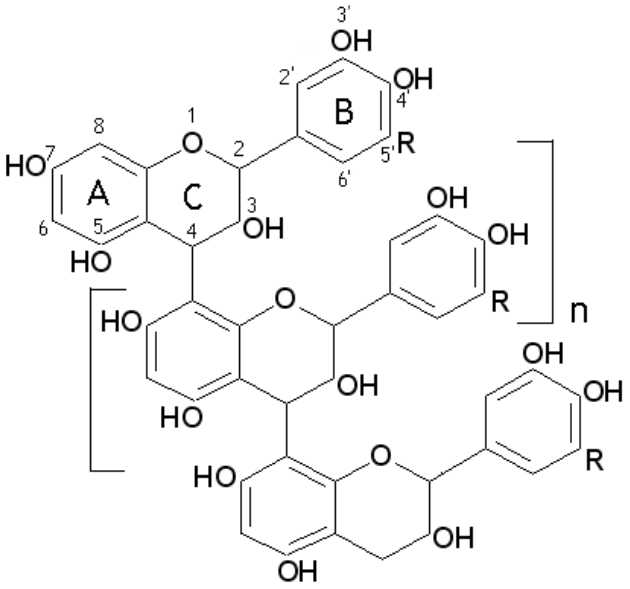

Fig. 1. Structure of two types of condensed tannins, procyanidins and prodelphinidins (for trimer n=1; for tetrameter n=2; etc.). Procyanidins (R=H) contain catechin and/or epicatechin (CE) subunits; prodelphinidins (R=OH) contain gallocatechin and/or epigallocatechin (GE) subunits.

The natural function of tannins

There are several theories of the natural function of tannins in plants. One of them is the defence theory, *i.e.* that tannins protect the plant against insects and herbivores. The suggested mechanism behind this is the supposition that feed tannins and saliva proteins form complexes in the mouth, thereby giving an unpleasant astringent "taste", and also that the tannins can affect digestion enzymes (Swain, 1979). Palo *et al.* (1993) found a negative correlation between tree height and concentration of phenolic substances (*i.e.* could be both tannins and non-tannin phenolics) in leaves of *Acacia tortilis*, *i.e.* leaves that could easily be reached by the animals contained higher concentration of phenolic substances. However, it is possible that the main purpose of tannins in plants is to protect them against invading fungi and bacteria, because tannin concentrations in many trees are found in the bark and not in the leaves. The tannin content represents up to 40% of the heartwood, which, without this protection, rapidly would get invaded by saprophytes (Swain, 1979).

Variation in tannin concentration

The amount of tannin in a plant depends both on the stage of development and the environment and there is a variation in tannin concentration both within and among species. Also, a reduction in tannin concentration with increased maturity is not necessarily a true reduction. It may also be an effect of decreased extractability (Hagerman & Butler, 1991). Singh *et al.* (1997) found the highest

sainfoin (*Onobrychis viciifolia*) tannin concentration in unfolded leaves and a decline in concentration during the subsequent leaf development. On the other hand, Skadhauge *et al*. (1997) found that in big trefoil (*Lotus uliginosus*, syn. *L. pedunculatus*), the tannin content in leaves increased with increased maturity, whereas the content decreased in the flowers.

Plants can produce phenolic substances as a response to environmental stress caused by nutrient deficiency, drought, high temperature, light intensity, and grazing (see Mueller-Harvey *et al.*, 1988). Lawler *et al*. (1997) found that the concentration of condensed tannins increased in the leaves of Eucalyptus when grown under nutrient deficiency or in strong light. The condensed tannin content of big trefoil was higher when grown in acid soils of low fertility than when grown in high fertility soils (Barry & Forss, 1983).

Tannin and protein interactions

For a long time, tannins were assumed to be generalists in the way of binding to proteins. As with other macromolecules, however, tannins are involved in specific interactions (Hagerman & Butler, 1981; Zhu *et al.*, 1997). The complex between tannins and proteins is dependent on the size and structure of both the protein and the tannin with a high affinity for proteins with a high proportion of proline. Proline-rich proteins (PRP) are found in the saliva of *e.g.* deer, but not in cattle and sheep (Austin *et al.,* 1989). The assumed function is that of protecting valuable feed proteins from the tannins. These proteins have an open tertiary structure, which facilitates the formation of hydrogen bonds between the hydroxyl groups of the tannin and the carbonyl oxygens of the peptide bonds in the protein. The binding between proteins and tannins seems to contain some kind of multiple binding site and thus the tannin-protein complex can be compared to a antibody-antigen binding (Hagerman & Butler, 1981). One of the attributes of tannins is the ability to precipitate proteins. Nevertheless, under certain conditions these can also form soluble complexes (Hagerman & Robbins, 1987). In a classic *in vitro* study, Jones & Mangan (1977) found that the most stable tannin-protein complex was in the pH interval 4.0–7.0, whereas 90% of the protein was released at pH 2.5 (simulating abomasal pH).

Tannins and microorganisms

In contrast to hydrolysable tannins, condensed tannins are resistant to microbial degradation (Makkar *et al.*, 1995). To protect themselves against tannins, microorganisms can excrete polymers with high affinity for tannins. Thereby the tannins can be prevented from binding to microbial enzymes (Scalbert, 1991). When cattle were fed a high-tannin birdsfoot trefoil diet, the rumen microbes excreted a protecting glycocalyx. When the animals were fed a low-tannin birdsfoot trefoil diet, no glycocalyx was detected (Chiquette *et al.*, 1988). Condensed tannins from sainfoin inhibited growth and protease activity for *Butyrivibrio fibrisolvens* and *Streptococcus bovis* by binding to cell wall polymers (Jones *et al.*, 1994). Studies of the inhibitory effect of flavanol monomers on microorganisms indicate that the number of hydroxyl groups on the “B”-ring (see

Fig. 1) affects the activity. Gallocathechin with three hydroxyl groups on the B-ring inhibited *Streptococcus*, *Clostridum*, *Proteus,* and *Staphylococcus* more than cathechin (two hydroxyl groups on the B-ring) did (Lowry, 1996). This is consistent with Molan *et al*. (2001) who found that rumen bacteria was inhibited more by condensed tannins from *Lotus pedunculatus* (predominantly prodelphinidin polymers) than by condensed tannins from *Lotus corniculatus* (predominantly procyanidin polymers).

Effect of tannins on protein metabolism

Tannins could affect the protein metabolism in ruminants in different ways (Woodward & Reed, 1997; Molan *et al*., 2001; Makkar, 2003). 1) Tannin-protein complexes could protect the protein from microbial degradation in the rumen. The complex may then be released when pH drops in the abomasum and the protein is hydrolysed by endogenous enzymes, releasing amino acids to be absorbed in the duodenum. 2) It is possible that tannins, depending on their concentration and structure, could both stimulatory and inhibitory to microbial protein synthesis. 3) Some animals (e.g. deer) on a high-tannin diet can protect themselves by excreting PRP in the saliva. Since tannins have high affinity for PRP, the feed protein can escape tannin binding. 4) The tannin-protein complex formed in the rumen is not released in the abomasum, or if released, binds again to proteins further down in the small intestine when pH rises again. That protein will be detected as fibre-bound N in the faeces. The last scenario gives low rumen fermentation, low urea concentration in the plasma, low N-retention, and high concentration of N in the faeces.

Aims of the thesis

The general aim of the thesis was to increase the knowledge of ruminal degradation of soluble protein and also to investigate the effect of condensed tannins on the degradation. The specific objectives were to:

- Investigate the effectiveness of different protein precipitation agents
- Screen common feedstuffs for their soluble protein content
- Estimate degradation rates of soluble proteins *in vitro*
- Estimate degradation rates of soluble proteins *in vivo*
- Investigate buffer N solubility and protein degradation in relation to tannin content

Material and methods

This thesis is based on several *in vitro* experiments and one *in vivo* experiment. The experiments were mainly conducted at Kungsängen Research Centre, Uppsala, Sweden but also at the Faculty Analytical Laboratory, Department of Agriculture, Reading University, UK and at the US Dairy Forage Research Center, Madison, Wisconsin, USA.

Procedures and chemical analyses are described in detail in the papers. Here the main emphasis is put on explaining the techniques used and some of the mechanisms involved.

Cultivation of tannin-containing plants (Paper III and IV)

Pure stands of birdsfoot trefoil (*Lotus corniculatus* L.) varieties were cultivated in single plots (Paper III) or with two replicates (Paper IV). In Paper IV, also sainfoin (*Onobrychis viciifolia* Scop.) and big trefoil (*L. uliginosus* Schkur., syn. *L. pedunculatus* Cav.) was cultivated. The birdsfoot trefoil variety referred to, as GA1 in Paper III is the same variety, referred to as Georgia One in Paper IV. Samples for chemical analyses were harvested when stands were in mid bloom. Varieties were collected in a first cut (Paper III) or in first and second cut during two consecutive years (Paper IV). All samples were immediately placed in an insulated cooler with ice and then frozen (-20ºC) within 30 min. Samples were freeze-dried and ground in a hammer mill to pass a 1-mm screen.

Two birdsfoot trefoil varieties with different tannin content, Norcen and Georgia One, were ensiled in laboratory-scale glass silos (Paper III). The herbage was dried outdoors, thinly spread on plastic sheets, for 4 or 8 h, then chopped in a

stationary cutter head (theoretical length 4 cm). Samples were ensiled without additives, stored at 25ºC for 200 days and then put in the freezer. Part of the material was thawed for pH determination and the rest freeze-dried.

Screened feed samples (Paper II)

Feeds commonly found in ruminant diets in Sweden were screened for soluble N and soluble protein N. The feeds were chosen to represent forages (fresh herbage, hay, and silage), cereals (wheat and barley), protein feeds (peas, lupine, linseed cake, soybean meal, and rapeseed) and industrial by-products (wheat distiller's grain). Fresh herbage, silage, and distiller's grain were freeze-dried, whereas hay, cereals, and protein feeds were dried at 60ºC for 16 h. All feeds were ground in a hammer mill to pass a 1-mm screen.

Soluble N and soluble protein N by Kjeldahl assays (Paper II and IV)

Total N was determined as Kjeldahl N according to the Nordic Committee on Food Analysis (1976). Buffer soluble N (BSN) was analysed by mixing feed and McDougall's buffer (MCD) and incubating for 1 h at 39ºC. After centrifugation, the supernatant was analysed for total N. Tanniniferous plants were also analysed by the BSN assay, extended from 1 to 24 h with and without polyethylene glycol (PEG, MW 3500). Incubation with PEG was first tested at the standard time of 1 h, but this resulted in lower BSN content in PEG samples than in samples without PEG. When incubations were run for 24 h, inclusion of 100 or 200 g PEG/g sample produced identical BSN concentrations. PEG has a particularly high affinity for tannins and can release protein from tannin-protein complexes, because tannins have a higher affinity for PEG than for proteins.

Buffer soluble protein N was analysed by trichloroacetic acid (TCA) precipitation. After centrifugation, the supernatants were analysed as for BSN. Buffer soluble protein N (referred to as Method 1 in Paper II) was calculated as the difference between BSN and TCA soluble N.

The ninhydrin assay (Paper I, II, and III)

Ninhydrin reacts with amino acids when heated under acidic conditions to produce a blue-purple complex with maximum absorption at 570 nm. Proline is an imino acid and the reaction product absorbs maximally at 440 nm. The reactivity of the α-amino-N can also be used in peptide and protein analysis after hydrolysis to liberate free amino acids. Ninhydrin also reacts with ammonia and, therefore, ammonia must either be removed or corrected for in samples such as silage and rumen fluid. This correction is easily performed in automated systems, where ammonia and α-amino acids can be quantified simultaneously.

Recovery of N in amino acids and protein (Paper I)

The recovery of N in 20 amino acids as α-amino-N and ammonia-N was investigated. Individual amino acids were analysed for α-amino acid-N and ammonia-N using the ninhydrin and phenol-hypochlorite assays with continuous-flow analysis as described by Broderick & Kang (1980). Tolerance of individual amino acids to hydrolysis was tested in 6 M HCl heated for 16 h at 110ºC.

The recovery of N as α-amino acid-N and ammonia-N upon hydrolysis of protein was investigated using casein and bovine serum albumin (BSA). Samples were dissolved in MCD and incubated for 1 h at 39ºC. Then, strained rumen fluid (SRF) or clarified rumen fluid (CRF; centrifuged at 30 000g) were added to the tubes and the samples centrifuged. Supernatants were hydrolysed and analysed for amino acid- and ammonia-N as described above.

Fractionation of N in feed and rumen fluid samples (Paper II)

Buffer soluble N fractions from feed and rumen samples were analysed for protein-, peptide-, amino acid- and ammonia-N (Paper II). Protein N was calculated as the difference between α-amino-N concentrations in hydrolysed BSN extracts or rumen fluid supernatants (total amino acid-N) and hydrolysed TCA extracts (peptide N + free amino acid-N). Peptide N was calculated as the difference between α-amino-N in hydrolysed and unhydrolysed TCA extracts (free amino acid-N) (Fig. 2).

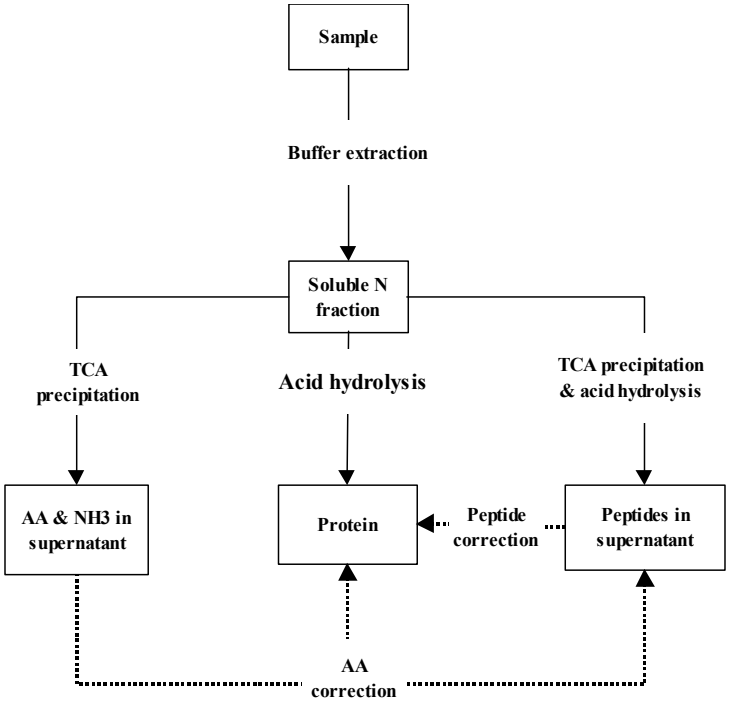

Fig. 2. Fractionation of buffer soluble N

Protein precipitation agents (Paper I)

The effect of TCA, perchloric acid (PCA), and tungstic acid (TA) on peptide recovery was investigated with two chemically-defined peptides, containing five and eight amino acid residues. The same protein precipitation agents were also investigated to assess the ability to detect feed peptides from *in vitro* rumen feed protein degradation. Casein, solvent extracted soybean meal, expeller extracted soybean meal, maize gluten meal, and lucerne hay were used as protein sources. SRF and proteins were prepared as described by Broderick *et al.* (2004). Protein precipitants were added to the rumen fluid prior to addition of the peptides to avoid microbial degradation. For the feed samples, rumen incubations were conducted for 2 h and then stopped by addition of the protein precipitants. Supernatants obtained from centrifugation of incubated feeds were analysed for amino acids and ammonia, with and without prior acid hydrolysis. The flow-injection procedure of Broderick *et al*. (2004) was used. Protein precipitant extracts of pure peptides, as well as aqueous solutions of the peptides without addition of protein precipitants (control samples) were hydrolysed and analysed for individual amino acids using ion exchange chromatography with ninhydrin detection.

Condensed tannins (Paper III and IV)

Tannins were extracted with aqueous acetone and analysed by the radial diffusion method (Hagerman, 1987) (Paper III and IV) and HCl-butanol assay (Porter *et al.*, 1986) (Paper III). In all cases, birdsfoot trefoil tannins purified on a Sephadex LH-20 column (Strumeyer & Malin, 1975) were used as standard.

Tannin structure and molecular weight was studied in Paper III. Thin layer chromatography (TLC) was used to screen the anthocyanidins produced by the HCl-butanol reaction (Mueller-Harvey, Reed & Hartley, 1987). The HCl-butanol reaction mixture was also examined for the relative proportions of delphinidin and cyanidin by high performance liquid chromatography (HPLC) according to Stewart, Mould & Mueller-Harvey (2000). The molecular weight of tannins was analysed by gel permeation chromatography (GPC) and MALDI-TOF mass spectrometry (Krueger *et al.*, 2000).

In vitro protein degradation (Paper II and III)

Two different ruminal *in vitro* methods were used. An inhibitor *in vitro* (IIV) method (Broderick, 1987) was used in Paper III to compare protein degradation in four birdsfoot trefoil varieties with differing tannin contents. The method was modified in Paper II, mainly by excluding the inhibitors (cloramphenicol and hydrazine sulphate). The inhibitors prevent microbial metabolism of amino acids and ammonia and, as a consequence, the release of these products can be used as an estimate of protein degradation. As bacteria are prevented from growing, the culture is slowly dying. Therefore, it is not possible to exclude that the bacteria are degrading the substrate at a reducing rate. By quantifying residual protein (Paper II), instead of amino acids and ammonia (Paper III), inhibitors were not required.

The reducing agent was also omitted in the modified *in vitro* method (Paper II). The use of reducing agents *in vitro* has been reported to both increase (Kohn & Allen, 1995) and decrease (Cotta & Hespell, 1986b) proteolytic activity. Rumen fluid was obtained for both methods from lactating dairy cows and filtered through cheesecloth to provide SRF. This was followed by a pre-incubation with carbohydrates for 3 h to reduce the concentration of ammonia and amino acids.

The alternative method was used to investigate degradation rates of soluble protein in 11 feeds (Paper II). Feeds were mixed with MCD and incubated similarly to the BSN samples after which rumen fluid was added to the protein solutions, giving a buffer/rumen fluid ratio of 1:2 (v/v). The same ratio was also used in the IIV method (Paper III). Tubes were incubated from 0 to 240 min (Paper II and III). Microbial activity was stopped by putting the tubes in a low-temperature slurry (Paper II) or by addition of TCA (Paper III) and tubes centrifuged (30 000g, 15 min, 4ºC). In the IIV method (Paper III), degradation was estimated from the build-up of amino acids and ammonia, whereas in the modified method (Paper II), soluble protein degradation was estimated from disappearance of TCA precipitated protein measured by bicinchoninic acid (BCA).

Calculation of effective protein degradation (EPD) in the rumen was done, assuming an outflow rate of 0.16/h for the buffer soluble proteins, using the following equation:

$EPD = [k_d/(k_d + k_p)] \times \exp(-k_p \times L)$, where
k_d = degradation rate (/h), k_p = passage rate (/h) and L = degradation lag (h).

In vivo soluble protein degradation (Paper II)

As part of a larger feeding trial, four rumen-fistulated dairy cows in mid-lactation (25.2±3.9 kg milk) were ruminally infused with either of two soluble proteins. The animals were fed silage (perennial ryegrass/red clover, 750/250 on a DM basis) *ad libitum* and 8 kg DM of concentrate (oats, 260; barley, 320; rapeseed cake, 120; peas, 300 g/kg).

To check ruminal concentrations of soluble N constituents after morning feeding, a pre-trial sampling was performed. Based on this trial, which showed that ruminal concentration of soluble protein and peptides had returned to baseline values (comparable to before the morning feeding) by 12.30, dosing experiments were started at 13.00. A pulse dose of 10.4 g soluble proteins, extracted from cold-pressed rapeseed cake or from peas, was given on two different days. Soluble protein was prepared as described for BSN. CoLiEDTA was used as liquid marker and prepared according to Udén, Colucci & van Soest, (1980). Each pulse dose consisted of 4 litres of soluble protein and marker and was infused into the rumen in less than 2 min. Rumen fluid was sampled before giving the pulse dose and at 0.5, 1, 1.5, 2, 2.5, 3, and 4 h after dosing. A PVC tubing (75 cm, 15-mm id.), plugged in one end and fitted with holes, was used for the sampling. Rumen samples were taken by inserting the tube through the fistula, *ca*. 50 cm down through the rumen mat. Rumen samples were immediately put in a low-temperature slurry and transferred to the lab for analyses of protein, peptides,

amino acids, and ammonia, using continuous-flow analysis (see Fractionation of N in feed and rumen fluid samples). Cobalt was determined by atomic absorption spectrometry.
To estimate rumen liquid volumes, total rumen evacuations were done twice in all cows the week after the pulse dose experiment. Rumen contents were weighed, thoroughly mixed, and sampled for DM.

Results

Soluble N and soluble protein N by Kjeldahl (Paper II and IV)

Buffer soluble N in 25 feeds (Paper II) ranged from 36 to 802 g/kg N. Lowest BSN content was found in maize gluten meal and heat-treated rapeseed meal. Highest BSN content was found in silages, peas, lupines, and cold-pressed rapeseed cake. Soluble protein- ranged from 0 to 874 g N/kg BSN. All silages contained very small concentrations of soluble protein N (0 to 71 g N/kg BSN). For protein feeds containing high concentrations of BSN, a large proportion was in the form of soluble protein. The soluble protein content in peas, lupines, and cold-pressed rapeseed cake were 874, 815, and 851 g N/kg BSN, respectively.

Buffer soluble N in tannin-containing plants ranged from 118 to 386 g/kg N in fresh-frozen samples (Paper IV). BSN concentrations increased in all samples when extraction time was extended from 1 to 24 h. In fresh samples the increase was approximately 26%, but in silage samples only 2%. When BSN was plotted against tannin content, there was a curvilinear relationship. Upon PEG addition, BSN contents in four out of five birdsfoot trefoil varieties became similar (499 to 542 g/kg N) whereas the BSN content in the fifth birdsfoot trefoil variety (Grassland Goldie), and in sainfoin and big trefoil, although increasing, never exceeded 400 g/kg N.

In silages, the BSN content was lower in Georgia One than in Norcen and an increased wilting time decreased BSN content in both varieties. Mean BSN contents (two wilting times) for Georgia One and Norcen were 580 and 690 g/kg N, respectively. The addition of PEG to the corresponding fresh-frozen herbage had a large impact on BSN for both varieties, although the effect was much larger for Georgia One. However, PEG had no effect on BSN in the Norcen silage, and only increased BSN content for Georgia One by 4%.

Recovery of N as amino acids and protein (Paper 1)

The recovery of N in individual amino acids, when analysed using ninhydrin detection, varied between 0.04 (proline) to 2.14 (glycine) with a mean value of 0.80. Ammonia was only detected for glutamine. When the amino acids were exposed to acid hydrolysis, the recovery of N varied between 0.04, with a mean of 0.81. Ammonia was detected for all amino acids and, including the ammonia-N in the recovery, yielded a mean value of 0.96. For the proteins, the recovery was 0.74 (amino acid-N plus ammonia-N) for both BSA and casein when MCD was the

solvent. When CRF and SRF were used as solvents, N recovery was lower for casein but only marginally lower for BSA.

Protein precipitant agents (Paper I)

No differences among protein precipitants were detected for peptide-bound amino acids and rankings were virtually identical for the five proteins. The recoveries were higher using TCA and PCA, compared to TA, for the chemically-defined peptides. Recovery of the peptide containing four amino acid (AA) residues was higher than that containing eight AA residues with all three precipitants.

Tannin content and structure (Paper III and IV)

Tannin content in birdsfoot trefoil varieties analysed by radial diffusion (total tannin assay) ranged between 3 and 10 g/kg DM (Paper III) or 4 and 18 g/kg DM (Paper IV). When analysed by HCl-butanol (CT-specific assay), varieties ranged between 2 and 17 g/kg DM (Paper III). In Paper III, varieties were grown in single plots and therefore varietal differences were not statistically analysed. In Paper III there were effect of year, harvest, and species/variety. In general, tannin contents were lower in Year 2 than in Year 1 and lower in Harvest 2 than in Harvest 1. Tannin contents in sainfoin (Year 1) and big trefoil were 21 to 22 and 25 g/DM (radial diffusion assay), respectively. Thus, tannin contents were higher in sainfoin and big trefoil than in any of the tested birdsfoot trefoil varieties (seven varieties in Paper III and five varieties in Paper IV).

Characterization of birdsfoot trefoil tannins (Paper III) by TLC showed presence of cyanidin and delphinidin in the HCl-butanol mixtures and for all varieties the intensity of cyanidin was stronger than for delphinidin. This feature was confirmed by HPLC, where delphinidin/cyanidin ratios varied from 16:84 to 33:67 with a mean of 25:75. Gel permeation chromatography revealed small varietal differences in the distribution of tannin molecular weights. Analysis by mass spectrometry of two varieties revealed two series of tannin oligomers. The first series consisted of well-resolved tannin tetramers (chain length, n=4) to hexamers (n=6), but also included oligomers up to the decamer (n=10). A second series showed the structure within these oligomers. Heteropolymers were more prominent than homopolymers. Homopolymers consisting of catechin/epicatechin units, *i.e.* procyanidins, were detected, whereas no homopolymers consisting of gallocatechin/epigallocatechin units, *i.e.* prodelphinidins, were detected.

In vitro protein degradation (Paper II and III)

There were significant differences in soluble protein degradation rates and estimated effective protein degradation (EPD) values using the modified i*n vitro* method (Paper II). Degradation rate was highest for casein (1.00 h^{-1}) and lowest for linseed cake (0.18 h^{-1}). Soybean meal, peas, and lupine were degraded at intermediate rates, 0.46, 0.39 and 0.34 h^{-1}, respectively. Three feeds—lupines, rapeseed meal, and linseed cake —displayed lag-phase phenomena (0.6–1.18 h). As a result, EPD values for rapeseed cake and rapeseed meal differed, despite the

same degradation rate. Among the fresh forages, degradation rate and EPD for red clover were significantly lower than for ryegrass, white clover, and birdsfoot trefoil (low-tannin variety, Norcen). The escape of soluble protein (1-EPD) ranged from 0.15 to 0.56 for concentrate feeds and from 0.25 to 0.33 for fresh herbage.

The extent of N degradation differed between Norcen and the other three birdsfoot trefoil varieties, estimated by the inhibitor *in vitro* method (Paper III). The proportion of N that was degraded after 4 h was highest in Norcen and lowest in Georgia One. Curve fitting also suggested that the degradation rates did not follow a single exponential function. However, due to the small number of data points (n=5), this suggestion was not investigated further. The correlation between tannin content (radial diffusion assay) and degraded N after 4 h was high (R^2=0.93).

In vivo soluble protein degradation (Paper II)

Pre-trial sampling, performed three weeks before the trial, showed similar peak values of soluble N fractions in two cows after morning feeding. Peak concentrations for soluble protein N, peptide N, amino acid-N and ammonia-N were 43, 137, 80, and 250 mg l^{-1}. Based on pulse doses of 10.6 g protein N, estimated rumen volumes from evacuations, and sampling prior to dosing (baseline concentration), protein concentrations were calculated to be 150 and 154 mg N l^{-1} rumen fluid for rapeseed cake and peas, respectively. However, 30 min after dosing only 33 and 26%, respectively, of the calculated protein N concentrations were recovered and protein concentrations had returned to baseline levels after 1 h. Peptide N concentrations increased insignificantly in both treatments, whereas ammonia concentration increased in the pea treatment but not in the rapeseed cake treatment.

Cobalt concentrations were also lower than expected. In 5 out of 8 cases, Co concentrations were higher after 1 h than after 0.5 h, indicating slow mixing. Excluding the 0.5-h value, ln-transformations yielded linear curves with a mean slope of 0.20 h^{-1} (i.e. fractional dilution rate). The purpose of the Co marker was to use it to correct the decline in protein concentration from saliva and drinking. As protein concentrations reached baseline levels within 1 h, no attempt was made to calculate degradation rates because of too few data points. The cows typically drank at the end of the sampling period, and the water intake (5-10 l) did not cause measurable fluctuations in Co concentrations.

General discussion

There was a large difference in soluble protein content among feedstuffs (Paper II). In cold-pressed rapeseed cake, peas, and lupines the BSN content ranged between 650 to 800 g/kg N, and 70% or more was in the form of protein. The BSN content in silage produced from five different crops – ryegrass, white clover, red clover, birdsfoot trefoil and maize – was also high, and ranged from

500 to 700 g/kg N. For silages, however, less then 10% of the BSN were in the form of protein. There are few reports on soluble protein content in silage. The results in Paper II agree with Choi *et al*. (2002a, b) and Nsereko *et al*. (1998) who also found low levels or no soluble protein in silages. In contrast, Volden, Mydland & Olaisen (2002) reported that around 30% of soluble non ammonia nitrogen (NAN) in the silage was in the form of protein. The differences between studies may be explained by choice of analytical method or due to factors other than the use of additives, since all four studies (Nsereko *et al.,* 1998; Choi *et al.,* 2000a, b; Volden, Mydland & Olaisen, 2002) used similar concentrations of formic acid or formic acid-based additives. Problems that can arise when soluble protein content is estimated by difference, both using the same and mixed assays are addressed in Paper I & II. Due to the large quantities of NPN in silage, a rapid and accurate method for determination of peptides rather than soluble protein in silage juice would be a very useful tool for evaluation of proteolysis in silage. This issue was recently addressed (Slottner, 2004), but no suitable method was found.

TCA and TA have been recommended as protein precipitation agents to separate buffer soluble N into NPN and true protein (Licitra, Hernadez & Van Soest, 1996), corresponding to the fractions A and B_1 in the Cornell Net Carbohydrate Protein Model (Sniffen *et al.,* 1992). Frequent citations of Licitra, Hernandez & Van Soest (1996) have contributed to a common view that TA and TCA have cut-offs of around 2–3 and 10 amino acid units, respectively. In Paper I, we found no differences among protein precipitants for the detection of feed peptides, but the recovery of two chemically-defined peptides was higher with TCA and PCA supernatants than with TA. However, use of TA resulted in a recovery of 0.66 for the eight-residue peptide, which conflicts with a cut-off of around 3 amino acids. Licitra, Hernandez & Van Soest (1996) based their statement of different cut-offs for TCA and TA on a publication by Greenberg & Shipe (1979). In this study the average peptide length was indeed 3–4 amino acids in TA supernatants, but the same peptide length was observed for TCA, picric acid and sulphosalicylic acid. Yvon, Chabenet & Pélissier (1989) investigated peptide solubility in a range of TCA concentrations (40–240 g/l). They found that solubility was not only a function of peptide length but also depended on peptide hydrophobicity. Based on the results in Paper I and published findings, it can be concluded that there is no distinct cut-off for peptide size using common protein precipitants. If a specific peptide cut-off is of importance, other techniques, *e.g.* molecular weight filters, may be more relevant.

Although the cows studied by Virtanen (1966) produced more than 4000 kg milk per year on a protein-free diet, such a diet would be inadequate for today's dairy cows. A daily milk protein yield of 1.5 kg (40–50 kg milk), cannot be sustained by microbial protein alone. The high-yielding dairy cow needs to be supplemented with dietary protein that escapes microbial degradation in the rumen. The estimation of microbial protein synthesis and dietary escape protein forms the basis for all modern ruminant evaluation systems. Protein evaluation using live animals is theoretically the best choice. *In vivo* trials, however, are expensive, labour-intensive and often require fistulation. Therefore alternative methods have been developed (Stern, Bach & Calsimiglia., 1997). The most widely used method is the *in sacco* method (Hvelplund & Weisbjerg, 2000) and

determinations by this method form the basis for several protein evaluation systems. The advantage with the *in sacco* method is that it is performed *in situ*. However the method suffers from several weaknesses, such as low reproducibility, microbial contamination of feed residues, and loss of small particles (see Hvelplund & Weisbjerg, 2000). Apart from the mentioned weaknesses with the *in sacco* method, the method cannot be used for estimations of soluble proteins. This is because solubility (disappearance from bags) is assumed to equal degradation in the *in sacco* method. Therefore, when soluble proteins were investigated, an alternative *in vitro* method had to be used in the present thesis (Paper II).

An ideal *in vitro* method should be simple, rapid and accurate. Many different *in vitro* methods have been developed that do not require fresh rumen fluid, *e.g.* use of fungal enzymes from *Streptomyces griseus* (Krishnamoorthy *et al*., 1983); mixtures of commercial proteases (Luchini, Broderick & Combs, 1996) and extracted enzymes from rumen content (Mahadevan, Sauer & Ehrle, 1987; Kohn & Allen, 1995). However, it is likely that the complex interactions involved in ruminal degradation of protein are best simulated by live ruminal microorganisms (Calsamiglia, Stern & Bach, 2000). *In vitro* ammonia production has been widely used as an indicator of ruminal protein degradation. Because microbes use ammonia for their own protein synthesis, degradation rates will be underestimated using this approach. Broderick (1987) developed a rumen *in vitro* system where chloramphenicol and hydrazine sulphate were added to inhibit microbial metabolism of amino acids and ammonia. This method was used in Paper III, where it successfully showed differences in N degradability among birdsfoot trefoil varieties. Still, the presence of inhibitors may result in reduced degradation rates due to end-product inhibition and a slowly starving microbial population.

In the soluble protein study (Paper II) we decided to estimate degradation rates based on residual protein instead of the end-products (amino acids and ammonia). With that approach we could exclude the inhibitors. We also excluded the reducing agent mercaptoethanol. Reducing agents are routinely used in *in vitro* systems to ensure anaerobic conditions. The primary reason for us to omit these agents was to exclude any effect on disulphide bonds in proteins rich in sulphur containing amino acids. In addition there are conflicting reports on the affect of reducing agents on proteolytic activity (Hazlewood, Jones & Mangan, 1981; Cotta & Hespel, 1986b; Kohn & Allen, 1995). In a preliminary study we investigated the effect of adding mercaptoethanol, titanium (III) citrate, or no reducing agent (control) on the degradation of BSA, casein and casein hydrolysate. The hypothesis was that if presence of low concentrations of reducing agents in the rumen incubations could reduce disulphide bonds in the substrates, this would be expressed as an increased degradation rate in BSA but not in casein and casein hydrolysate. Degradation rates of slowly degrading soluble proteins, such as BSA, were increased three to eightfold when treated with reducing agents to break disulphide bridges (Mahadevan, Erfle & Sauer, 1980; Nugent *et al.,* 1983), while Fraction 1 leaf protein, a protein without known disulphide bonds, did not respond to the treatment (Nugent *et al.,* 1983). Like Fraction 1 leaf protein, casein has few or no disulphide bonds, and is rapidly degraded (Mangan, 1972) Interestingly, mercaptoethanol, titanium (III) citrate and control produced similar degradation profiles by the IIV method for casein and casein hydrolysate, whereas BSA

degradation profiles were similar up to 2 hours, and then increased in the control treatment, as compared to the other treatments (Hedqvist, unpublished). Recently Mould, Morgan & Kliem (2004) reported very similar cumulative gas profiles *in vitro* with and without a reducing agent. This is absolutely an area that needs further investigations.

The soluble proteins were degraded at different rates using the modified *in vitro* method (Paper II). The much higher degradation rate observed for the control sample (casein), compared to other soluble proteins, is consistent with other *in vitro* and *in vivo* studies (Mangan, 1972; Mahadevan, Erfle & Sauer, 1980; Broderick, 1987). We estimated the degradation rate for casein to be 1.0 h^{-1}. In the IIV method, casein degradation rates have been reported to be 0.294 h^{-1}, when only amino acids and ammonia were quantified, or 0.373 h^{-1}, when peptides were included (Broderick *et al.*, 2004). Modification of the IIV method, using $^{15}NH3$-enriched rumen inocula and no inhibitors, gave a higher degradation rate, 0.540 h^{-1} (Hristov & Broderick, 1994). By applying Michaelis-Menten saturation kinetics on IIV data, Broderick & Clayton (1992) estimated a degradation rate of 0.989 h^{-1}, which is very close to the result in the soluble protein study (Paper II). In the calculation of EPD, we applied a liquid passage rate of 0.16 h^{-1}. This value falls into the range (0.14 to 0.23 h^{-1}) reported in lactating cows (Chen, Sniffen & Russell, 1987; Hristov & Broderick, 1996, Volden, Mydland & Olaisen (2002), Choi *et al.*, 2002b; Reynal & Broderick, 2003). Calculated escape of soluble protein (1-EPD) ranged from 0.15 to 0.56 in concentrate feeds and from 0.25 to 0.33 in fresh herbage. In the official feeding recommendations in Sweden (Spörndly, 2003), EPD values are calculated from *in sacco* estimations, without correction for loss of small particle or solubles. Standard EPD values for soybean meal, rapeseed meal and peas are 0.64, 0.72, and 0.80, respectively (Spörndly, 2003). By correcting the *in sacco* data that corresponded to these values for BSN content (estimated in Paper II), EPD values for the insoluble N fraction were calculated. These values were then combined with EPD values for the soluble N fraction to yield an adjusted EPD. The adjusted EPD values for soybean meal, rapeseed meal and peas were 0.61, 0.62 and 0.72, respectively. For rapeseed meal, the decrease in EPD from 0.72 to 0.62 resulted in an increased calculated value for amino acids absorbable in the small intestine (AAT; Madsen, 1985; Spörndly, 2003) from 112 to 140 g/kg DM (Hedqvist, 2004).

All *in vitro* estimates need to be validated against *in vivo* estimates. However, in the soluble protein study (Paper II), where peas or cold-pressed rapeseed cake were given as a pulse dose to dairy cows, we could not calculate any degradations rates. Both proteins were rapidly degraded and, within one hour, ruminal protein concentrations had returned to baseline levels. This low recovery of the proteins probably derived from at least two sources: a slow mixing of the pulse dose in the rumen and too long sampling intervals. The first issue has been addressed by Warner & Stacy (1968), who concluded that mixing of the soluble marker $^{51}CrEDTA$ in sheep was not complete, even 1.5 h post dosing. Furthermore, Teeter & Owens (1983) found that the time from marker administration to peak concentration in the rumen of fistulated steers ranged from 0.20 to 1.77 h. There are few reports on soluble protein degradation *in vivo* available for validation of the *in vitro* data (Paper II). Volden, Mydland & Olaisen (2002) gave pulse doses

of soluble extracts from grass and silage to dairy cows. The degradation rate decreased linearly with increasing dosage with an average degradation rate of soluble protein-N of 2.14 h^{-1}. This is 4–5 times higher than the estimates for fresh herbage in Paper I. Mangan (1972) estimated the degradation rate of casein to 1.93-7.70 h^{-1}. As in the soluble protein study (Paper II), both these studies reported possible incomplete mixing of the pulse dose at early sampling times. Broderick (1978) used a pump that circulated rumen contents to by-pass the mixing problem. He estimated the degradation rate for casein to 0.46 h^{-1}, which is much lower than the values reported by Mangan (1972) and about half of our estimates *in vitro* (Paper II). Based on these conflicting results, it is clear that more *in vivo* studies on soluble proteins are needed and also that the mixing problem need to be solved.

In the first tannin study (Paper III), tannin contents in seven birdsfoot trefoil varieties ranged between 3 to 10 g/kg DM when measured with the radial diffusion method (Hagerman, 1987), which detects both hydrolysable and condensed tannins. Using the HCl-butanol method by Porter, Hrstich & Chan (1986) that is specific for condensed tannins (CT), somewhat higher concentrations were found (2–10 g/kg DM). Despite these rather low tannin concentrations, we found differences in *in vitro* ruminal protein degradation. However, results found *in vitro* cannot automatically be translated into animal performance. Therefore we screened other birdsfoot trefoil varieties and also other tannin-containing species with the aim of finding varieties with somewhat higher tannin content. We found that birdsfoot trefoil varieties from New Zealand (Grasslands Goldie) and South America (Draco, San Gabriele) had higher tannin content than North American varieties Fergus, Norcen, Georgia One), but they were also less winter hardy (Hedqvist, Murphy & Nilsdotter-Linde, 2002). The most promising birdsfoot trefoil variety tested in Sweden so far (Paper III, Hedqvist, Murphy & Nilsdotter-Linde, 2002) is probably Oberhaunstaedter. The tannin content in Oberhaunstaedter was somewhat lower than in Grasslands Goldie (Paper IV), but the agronomic performance was better (unpublished data). Experimental trials with Grassland Goldie have demonstrated improvements in milk production, wool growth and live weight gain in sheep (reviewed by Waghorn *et al.*, 1998) and increased milk production in dairy cows (Woodward *et al.*, 1999). In Paper IV we found significant differences between species/varieties in BSN content, and when regressed on tannin content, solubility decreased curvilinearly with increasing tannin content. All herbage samples increased in BSN upon PEG addition and, except for the birdsfoot trefoil variety Grasslands Goldie, sainfoin and big trefoil, the values became similar. Looking at net release of BSN (with and without PEG addition), the net release is higher for Oberhaunstaedter than for Grassland Goldie, sainfoin and big trefoil. If we speculate that BSN released by PEG addition corresponds to tannin-bound protein, resistant to ruminal degradation but released in the abomasum, Oberhaunstaedter would contribute most. The condensed tannins in birdsfoot trefoil are known to increase absorption of amino acids in the small intestine, while this is not always true for condensed tannins in big trefoil and sainfoin (Waghorn *et al*., 1998).

In Sweden, the grazing period is rather short (3–5 months) and therefore the effect of tannins in silage may be more interesting than in fresh herbage.

Interestingly, tannin content and wilting time cumulatively decreased BSN content in the silage. In a recent study by Hymes-Fecht *et al.* (2004) birdsfoot trefoil silages, containing three different levels of tannins, were compared to lucerne and red clover silages in dairy cows. DM intake was similar among diets, but milk and protein yields were higher for normal- and high-tannin silages compared to the others. Also, lower milk urea nitrogen concentrations were found with these two diets. These observations therefore indicate a more efficient N utilization of these silages, due to their tannin contents.

Conclusions

- Silages contained virtually no soluble protein whereas for peas, lupines, and cold-pressed rapeseed cake, the major part of the soluble N was in the form of protein.

- The degradation rate for soluble proteins ranged from 0.18 h^{-1} to 1.0 h^{-1}. The difference in degradation rate showed that degradation of soluble proteins is far from instantaneous. Therefore, the *in sacco* method should not be used for feeds high in soluble proteins, since in that method, solubility is assumed to equal degradation

- There is no distinct cut-off for peptide size when using protein precipitation agents, such as TCA, PCA and TA.

- N solubility and degradability were correlated to tannin content

- There was a cumulative affect of tannins and wilting time on buffer soluble N in silage.

Populärvetenskaplig sammanfattning

Mjölkkor behöver stora mängder protein till mjölkprotein. Proteinet kan komma från två källor; fodret och mikroorganismer. Den här avhandlingen fokuserar på foderproteinet, olika metoder att mäta och förstå dess nedbrytning. Eftersom det både är kostsamt och innebär en miljöbelastning att överutfodra med protein studerades faktorer som kan påverka proteinnedbrytningen. Gamla växtkunskaper har uppdaterats kring den vanliga växten käringtand (*Lotus corniculatus*) som inte bara har ett högt proteininnehåll utan även kan fördröja proteinnedbrytningen.

Nedbrytning och värdering av foderprotein

Det protein som kommer med fodret beräknas vanligtvis genom att fodret analyseras för sitt kväveainnehåll. Därefter multipliceras kvävehalten med 6,25 för att få fram råproteinhalten. Faktorn 6,25 kommer från antagandet att protein i genomsnitt innehåller 16 g kväve per 100 g protein. I verkligheten kommer andelen kväve variera beroende på vilka aminosyror som ingår i proteinet. Allt kväve som finns i fodret ingår inte heller i protein. Fodermedel innehåller också andra kväveföreningar som peptider, aminosyror, ammoniak, nukleotider etc. Idisslare lever i symbios med våmmens mikroorganismer; bakterier, protozoer och svampar. Nedbrytningen av foderprotein i våmmen beror på flera faktorer. Beroende på proteinets kemiska och fysikaliska egenskaper, kommer det att vara mer eller mindre svårnedbrutet. Generellt brukar man säga att proteiner som är lösliga i våmmen bryts ned fortare än de som är olösliga. Andelen protein som bryts ned i våmmen kan beskrivas med ekvationen

$$EPD = Kd/(Kd + Kp),$$

där EPD står för effektiv proteinnedbrytning (från engelskans effective protein degradation), Kd är nedbrytningskonstanten för ett specifikt protein (per timme) och Kp är utflödeskonstanten (per timme). EPD blir då en fraktion mellan 0 och 1 eller om den multipliceras med 100, mellan 0 och 100%.

Den vanligaste metoden för att värdera foderproteiners nebrytning är den s.k. *in sacco*-metoden. *In sacco* betyder ”i påse” på latin och metoden kallas även (nylon)påsmetoden. Den innebär att foderprov inkuberas i nylonpåsar i våmmen. Djuren har därför en öppning, en fistel, till våmmen på vänster sida av buken. Nylonpåsarna får stanna olika lång tid i våmmen, varefter de tas ut och sköljs. Nylonvävens hål (ca 35 mikrometer) ska vara tillräckligt stora för att mikroorganismer ska kunna komma in i påsen, samtidigt som endast nedbrutet fodermaterial ska kunna komma ut ur påsen. Lösliga partiklar som försvinner ur påsen på en gång anses ha en momentan nedbrytning. Fodermedel med stor andel lösligt protein får därför höga EPD-värden.

Nedbrytning av lösliga proteiner i våmmen

Lupiner och ärter innehåller mycket lösligt protein. Vi utvecklade en alternativ metod till *in sacco*-metoden för att kunna mäta nedbrytningen för dessa och andra lösliga proteiner. Vi undersökte proteinerna in vitro (i provrör) med våmvätska. Det visade sig att proteinerna bröts ned olika fort. Genom att sammanfoga dessa värden med de vanliga EPD-värdena kan man få ett mått på nedbrytningen av både lösliga och olösliga proteiner.

Tanniner minskar proteinets löslighet

Om proteiner bryts ned för snabbt i våmmen till ammoniak hinner inte mikroorganismerna ta hand om all ammoniak. Den kommer då till stor utsöndras i urinen och därmed gå till spillo. Det finns olika sätt att minska proteinnedbrytningen i våmmen. Proteinstrukturen kan förändras och proteinet göras mindre tillgängligt för mikroorganismerna genom värmebehandling eller genom tanniner som finns i vissa växter. Tanniner är ett samlingsnamn för en grupp av vattenlösliga ämnen som kan binda till proteiner och andra makromolekyler. På svenska kallas tanniner även för garvsyror. Garvsyror användes förr när man skulle bereda skinn av råhudar. Genom bindningen mellan tanninerna och proteinen blev skinnen mjuka och motståndskraftiga mot bakterier och andra mikroorganismer. Egenskapen som utnyttjas vid garvning är samma funktion som man antar att tanninerna har i växter, nämligen ett skydd mot insekter och andra skadedjur. För växten är tanninerna också ett skydd mot betande djur. När djuren äter växter som innehåller tanniner, frigörs tanninerna under tuggningen. Genom att binda till proteiner som finns i saliven bildas olösliga komplex som ger en sträv upplevelse. Det är samma upplevelse som vi kan få när vi dricker ett rödvin med ”sträva tanniner”.

Kondenserade och hydrolyserbara tanniner

Tanniner brukar delas in i två huvudtyper; hydrolyserbara och kondenserade. De hydrolyserbara tanninerna kan som namnet antyder hydrolyseras, dvs spjälkas, både på kemisk väg och av mikroorganismerna i våmmen. I och med att molekylerna spjälkas blir de mindre och kan då tas upp i tunntarmen. De kondenserade tanninerna kan inte spjälkas i någon vidare utsträckning av mikroorganismerna utan passerar ut med träcken utan att att absorberas. Tannin-proteinkomplexet är som stabilast under de pH-nivåer som råder i våmmen och proteinet kan därför delvis undgå nedbrytning i våmmen. Komplexet är dock känsligt för pH-sänkning och kommer därför att till stor del spjälkas i löpmagen, där pH sjunker till följd av saltsyraproduktion. Efter löpmagen kommer proteinet till tunntarmen och där kan det spjälkas av kroppsegna enzymer. Det protein som inte snabbt spjälkas riskerar att åter bindas till det tannin som frigjordes i löpmagen. Tannin-proteinkomplex som inte upplöses i löpmagen, alternativt återbildas i tunntarmens senare del, kommer att att utsöndas i träcken. Det gäller att hitta en balans vid utfodring av fodermedel som innehåller tanniner. Minskningen i urinkväve får inte motsvaras av en lika stor ökning av kväve i träcken.

Låga tanninhalter påverkar

I det första svenska försöket om tanniner i käringtand undersöktes tanninhalten med två olika metoder i sju olika sorter. Förutom mätning av koncentrationen, undersöktes också strukturen på tanninerna. Alla sorterna innehöll ganska låga tanninalter (< 2% av torrsubstansen). Trots de låga halterna fann vi skillnader i proteinnedbrytning mellan sorterna när de undersöktes *in vitro* med våmvätska.

Det var intressant eftersom det tidigare ansetts att det krävs högre halter för att få en effekt.

Mjölkkor utfodras under större delen av året med konserverat foder, som kan vara i form av hö eller ensilage. En stor del av proteinet bryts ned i ensilaget av växtenzymer som frigörs i grödan vid slåtter. Vid ensilering av två sorters käringtand fann vi att sorten Georgia One hade lägre andel lösligt kväve än sorten Norcen och att en längre förtorkning (högre torrsubstanshalt) också gav en lägre löslighet. Kombinationen av naturliga tanniner i grödan och förtorkning vid ensilering kan bidra till att proteinet i ensilaget inte bryts ned lika mycket under ensileringsprocessen. På så sätt kan en större del av proteinet användas av mjölkorna.

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