MANIPULATION OF RUMEN AND SILAGE FERMENTATION

BY

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ABSTRACT

The effects of oxidants and surfactant additives on silage and forage fermentation were studied. First, the effects of two nonionic surfactants, Tweens 60 and 80, on rumen protease and cellulase activity and in vivo digestibility was investigated. At 0.25% of either Tweens 60 or 80 in the in vitro reaction mixture, both protease and cellulase activity were enhanced ($P < .05$). The inclusion of 0.5% Tween 80 in the grass hay diet fed to sheep, increased dry matter intake (DMI) by about 40%, and apparent nutrient digestibility by up to 10 percentage units. Hence, the potential benefits of Tween 80 in ruminant forage rations were established.

Secondly, the effect of Teepol HB7 on mixed rumen protease and cellulase activity, and digestibility. Up to 0.3% Teepol increased SH masking significantly, without adverse effects on cellulose degradation and microbial protein adsorption. The effective degradation of canola meal protein was decreased ($P < .05$), while the in vitro degradation of ruminal fluid soluble protein was reduced by more than 40% with the addition of 0.1% Teepol. In vivo rumen ammonia nitrogen concentration also dropped ($P < .05$). However, DMI and apparent nutrient digestibility were not affected ($P < .05$).

Thirdly, the proteases of orchard grass were characterized according to their mechanistic reactions and pH dependence. The rate of proteolysis (μmol glycine equivalent/g/h) declined with increasing acidity (pH 7-4) and ensiling period.

Phenylmethanesulfonylfluoride and p-chloromercuribenzoate inhibited proteolysis by up to 90% in the first 5 d after ensiling. It was concluded that orchard grass proteases are primarily thiol in nature. Lastly, the effect of thiol reagents on orchard grass silage fermentation was evaluated on the same material as in the latter experiment. Tween 80 resulted in a rapid decline in pH, but the final TCA-precipitable protein was higher ($P < .05$) than in the control. The true protein content and pH of the other anionic-surfactant based and KIO_3 treatments was higher ($P < .05$) than that of the control. It was concluded that Tween 80 could be used to increase silage fermentation and Teepol HB7 or KIO_3 to reduce proteolysis.

Keywords: rumen, silage, protein, thiol, surfactant

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LIST OF ABBREVIATIONS

BCA = bicinchoninic acid

BW = body weight

CHO = water soluble carbohydrates

CYS = cysteine

CYS-SH = SH = thiol group of cysteine

DMI = dry matter intake

HLB = hydrophile lipophile balance

KIO = KIO_3 = potassium iodate

Orchard grass = Dactylis glomerata

PCMB = p-chloromercuribenzoate

PMSF = phenylmethanesulfonylfluoride

SDS = sodium dodecylsulphate

SS = CYS-CYS = cystine = disulfides

TCA = trichloroacetic acid

Tp = Teepol = Teepol HB7 = sodium alkylsulfate

T6 = Tween 60 = polyoxyethylenesorbitan monostearate

T8 = Tween 80 = polyoxyethylenesorbitan monooleate

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Chapter 1

INTRODUCTION AND OVERVIEW

So far, complete expression of the cow's genetic milk production potential has not been realized, mainly due to the ruminal digestive inefficiency. Most ruminant nutritionists believe that high-yielding cows fail to produce to their genetic capacity as a result of inadequate supply of key nutrients. Consequently, increasing digestive efficiency through manipulation of the degradative processes in the rumen presents the greatest challenge to today's ruminant nutritionist. This challenge is also borne by the farmer who has to generate a living by working with an imperfect biological system in a competitive and demanding socio-economic environment. His success therefore lies in increasing efficiency at all stages of milk production, including feeding. Hence, research has concentrated on technologies that efficiently deliver the nutrients of the formulated rations to the cow for milk synthesis.

Feed processing technologies hitherto proposed, e.g., grinding and heat treatment, tannin and formaldehyde incorporation, are either too tedious, too expensive or are hazardous to the workers. These technologies have persistently focused on manipulating the bulky feed, ignoring the fact that the digesta matrix is a substrate - enzyme (microbial) interface with charge-mediated reactions. It is perhaps cheaper to manipulate the microbial enzymes than the bulky feed. In demand therefore, are new nutritional technologies that are cheap and versatile. These interventions should enhance the nutritive value of the feed by increasing overall digestibility while optimizing the digesta reactions.

In dairy production, forages constitute a major portion of the feed base. Polysaccharides, viz. pectins, hemicelluloses, celluloses and small amounts of sugars, are the main components of fibrous forage. The slow rate of fibre degradation provides a barrier to the attack on other cell components, thus slowing down the nutrient release process. In contrast, the high quality storage proteins function as food reserves in oilseed meals. As such, they must be readily mobilized when required by the plant. Hence, they are easily degraded by plant hydrolytic activities. Similarly they are highly susceptible to the degradative processes of rumen microbes. Their degradation is accompanied by deamination, an inefficient energy releasing process that ultimately results in ammonia production. Similarly, silage protein preservation is limited by the extensive degradation that occurs during ensiling. As a result of plant protease activities and bacterial deaminases, up to 60% proteolysis may occur in the first week of ensiling.

Protease inhibition studies have shown that the predominant type of protease present in rumen contents, mixed rumen bacteria and extracted capsular enzymes, is a cysteine (CYS-SH) type, sensitive to p-chloromercuribenzoate (PCMB, 56-89% inhibition, Wallace and Cotta, 1988). Other types of activity are also present, but are more variable. These include phenylmethanesulphonyl fluoride (PMSF)-sensitive serine protease (present at 0-41% of total activity), metalloprotease (9-30%) and aspartic protease (2-15%). CYS-SH proteases have also been intimately associated with plant protease hydrolases (Singh, 1962; Glazer and Smith, 1971; McKersie, 1981; Csoma and Polgar, 1984). From the foregoing evidence, the importance of the SH groups in

ruminal protein and silage fermentation was firmly established. Consequently, it was hypothesized that chemical changes on the catalytic SH groups would provide an opportunity to manipulate fermentative processes on ensiling or in the rumen. Increasing ruminal protein bypass without hampering total tract digestibility could entail masking SH groups of the ruminal microbial enzymes, if these groups are not involved in cellulose degradation. Clarke and Yaguchi, (1985) showed that the catalytic site of cellulases consists of glutamic and aspartic acids that act in a manner analogous to lysozyme and not SH residues. Hence, the properties of CYS-SH proteases could be manipulated without adverse effects on ruminal fibre digestion. However, this necessitates a clear understanding of the properties of the SH groups in proteins.

Fortunately, from various biochemical reports, the properties of SH groups in proteins have been explored. The most studied is papain. Experiments with papain showed that its activity is directly proportional to the content of the SH groups (Klein and Kirsch, 1969 a,b). Papain could also be inactivated by forming disulfide bonds. Further, Heitmann (1968) observed that the insertion of CYS-SH groups of proteins into anionic micelles is accompanied by their masking, i.e. by a sharp decrease in their reactivity. Similar observations were made by Sasago et al. (1963) on milk CYS-SH content in the presence of 0.25% sodium dodecylsulphate (SDS). At low level (< 0.5%), SDS was also found to increase the dough mixing time of bread (Danno and Hoseney, 1982), a fact attributed to the masking of CYS-SH residues of gluten. Another dough conditioner, potassium iodate is used to enhance disulfide formation via thiol-disulfide interchange (Hird and Yates, 1961; Tsen and Bushuk, 1963; Tsen,

1966). It is evident that these products or their substitutes could be useful in the manipulation of SH residues during rumen and silage fermentation.

Enhancement of microbial fermentation through the use of surfactants has acquired successful and wide applications in biogas and industrial glucose production bioreactors (Castanon and Wilke, 1981; Madamwar et al., 1991; Helle et al., 1993). By monitoring sugar production from cellulose mixed with 0.1% Tween 80, Castanon and Wilke (1981) were able to increase the rate of cellulase efficiency by 33% in anaerobic reactors. Other studies have reported the ability of Tween 80 to increase cellulase stability (Kim et al., 1982; Helle et al., 1993), lipolysis (Hazlewood and Dawson, 1975) and viable bacterial count (Akin, 1980; Dehority and Grubb, 1980). In addition, Triggle (1970) and Machtiger and Fox (1973) established that cell and bacterial membrane associated enzymes could be activated by Tween 80. Needless to mention that the potential application of surfactants in the feeding of fibrous forages and silage preservation is tremendous. The preservation and utilization of these forages are limited by inadequate lactic acid production, and slow rates of fermentation, respectively.

The success of any additive is normally dependent on its effectiveness, cost and its potential hazardous effect. As was shown above, very low levels of surfactants have been employed in industrial reactors. Currently, food grade surfactants are inexpensive and readily available. In fact, Tween-80 is currently used in the confectionery and ice-cream industry. This approach differs from hitherto proposed methods (Broderick et al., 1991; Jouany, 1994) for "protecting" proteins from ruminal degradation. No documented attempts have been made to manipulate rumen or silage protein

fermentation via partial inhibition of proteolytic enzymes, or unmasking activity to increase the digestion of fibrous forages. Hence, the main goal of this study was to exploit surfactant additives in maximizing feed utilization of high quality feed proteins and fibrous forages.

To achieve this goal the following specific objectives were identified;

a) To study the effects of Tween 80 and Teepol HB7 on the activity characteristics of rumen proteases and cellulases.

b) To determine the effects of the above surfactants on feed intake and digestibility in ruminants.

c) To determine the effects of these additives and thiol oxidants on silage fermentation.

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Chapter 2

REVIEW OF LITERATURE

2.1 Feeding the modern dairy cow

Global trading laws, the farmers economic survival and environmental concerns are increasingly exerting pressures for improved farm efficiency. No where is this pressure being felt more than in the nutrition area, as feeds consume a major portion of the farm budget. In addition, it is believed that many cows fail to produce to their genetic capacity because of inadequate supply of key nutrients. Clark (1975) proposed that this shortage may result from limited appetite, physical capacity of the animal or inability of the rumen microbial system and/or tissues of the animal to generate sufficient quantities of precursors to meet the demands of the mammary glands for maximum synthesis of milk. Hence, it is undeniable that it is becoming increasingly difficult to meet specific nutrient requirements of the dairy cow as the milk productivity per cow increases. Consequently, there is need to increase milk production per unit of feed consumed by the cow.

Studies on postruminal infusion of intact proteins (casein), amino acids, and/or glucose have unveiled marked increases in milk yield and composition, particularly milk -protein content (Broderick *et al.*, 1991; Clark, 1975). With the conventional feeding of high quality protein supplements, extensive ruminal protein breakdown to amino acids results. Since microbial growth is not synchronized with the rapid protein degradation, substantial amounts of amino acids are deaminated to supply energy rather than being used for microbial protein synthesis. In addition, approximately 30% of the

bacterial protein produced in the rumen is also degraded therein (Chalupa, 1975). Thus, controlling the rate and extent of degradation of dietary protein to balance the protein supply from microbial synthesis is of great interest to the nutrition of the modern dairy cow.

2.2 Measurements of dietary protein degradation in the rumen

The potential for ruminal protein bypass of feedstuffs can be measured by several methods. These include solubility in various solvents, rate of hydrolysis using a variety of proteases, ammonia release, and in situ disappearance (Broderick *et al.*, 1991; Broderick, 1994). Solubility measurements are a simple means of ranking feeds based on their dispersion in a buffer. Measurements of protein solubility have been discussed by Broderick (1987). However, attempts to equate protein degradability and escape protein with insolubility have not been successful. Bovine serum albumin and ovalbumin are both water soluble yet they are degraded in the rumen much more slowly than casein or other soluble proteins (Mahadevan *et al.*, 1980). Treatment of bovine serum albumin with dithiothreitol to break some of the disulfide bridges crosslinking the protein, markedly increased its rate of degradation. Similarly, the rate of degradation of the insoluble fraction of diazo-fishmeal was increased nearly ten fold by treatment with mercaptoethanol. Thus, the number of disulfide bridges and the tertiary structure of a protein in general are obviously very important factors in determining its degradability. The availability of end groups has also been indicated to be important determinants of protein susceptibility to degradation.

A common approach for estimating protein degradability involves incubation of the test protein with rumen fluid and measurement of the subsequent ammonia

production (Broderick, 1987). Though simple, this method has the disadvantages that; microbial growth and ammonia uptake may occur simultaneously with protein degradation and ammonia release. Also, daily variation in ruminal ammonia concentration of donor animals and the production of ammonia from sources other than feed protein limit this method. Broderick (1987) suggested using hydrazine (an inhibitor of end-product metabolism) to overcome microbial growth and ammonia uptake.

Other researchers have substituted commercial proteases for rumen protease in their procedures. These proteases should have specificities similar to ruminal proteases. Mahadevan *et al.* (1987) has described a procedure for harvesting and preparation of rumen proteases for use in degradation trials. Broderick *et al.* (1988) compared the in vitro and the in situ estimates of ruminal degradation rates. They observed that, (a) in vitro degradation rates were more comparable with reported in vivo rates of ruminal passage and extents of degradation and, (b) the in situ procedure was more precise for slowly degraded or insoluble proteins.

For verification of the results obtained from in vitro and in situ studies, in vivo studies are usually carried out. In addition to being expensive, these studies are complicated by lack of accurate and reliable methods of estimating the microbial protein fraction and the endogenous protein secretions. Nonetheless, these techniques are the basis for comparing the degradability of the protein fractions of ruminant diets.

2.3 Microbial colonization in the rumen

Rumen bacteria, fungi and protozoa colonize all plant parts, mainly via the epidermal lesions. However, in the leaves, colonization through stomata is thought to

production (Broderick, 1987). Though simple, this method has the disadvantages that; microbial growth and ammonia uptake may occur simultaneously with protein degradation and ammonia release. Also, daily variation in ruminal ammonia concentration of donor animals and the production of ammonia from sources other than feed protein limit this method. Broderick (1987) suggested using hydrazine (an inhibitor of end-product metabolism) to overcome microbial growth and ammonia uptake.

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2.3 Microbial colonization in the rumen

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be particularly important (Cheng *et al.*, 1991). As an initial step in the degradation process, the rumen microbes must become attached to cell walls. The mechanism of microbial attachment to the feed particles, especially cellulose, is thought to involve cell surface-associated enzymes, adhesins or possibly, non-specific ionic interaction (Cheng *et al.*, 1991). Evidence using isotope-labelled protein suggests that even soluble proteins are adsorbed on to microbial surfaces while they undergo hydrolysis (Hobson and Wallace, 1982). Bar-Or (1990) brought out evidence indicating the involvement of microbial surface proteins in cell surface hydrophobicity. He indicated that p-chloromercuribenzoate (PCMB- SH blocking agent) resulted in a marked decrease in cell surface hydrophobicity in Vibrio proteolytica. Indeed, Wallace (1985) showed that adsorption of soluble proteins to the surfaces of rumen bacteria is probably an integral part of the catalytic process rather than a separate phenomenon. Further, he postulated that manipulation of adsorption may therefore not be separable from the manipulation of proteolysis itself. It is known that membrane proteins are not only highly hydrophobic but are also high in SH content (Levitt, 1980). Appreciable evidence exists suggesting that the nonpolar surroundings of SH groups can favour the binding of hydrophobic reagents. Further investigations seem warranted to resolve whether SH groups and/or their normally hydrophobic environments are indeed involved in adhesion of rumen bacteria to feed protein particles.

2.4 Mechanistic classification of proteolytic enzymes

In earlier days, proteases were classified according to molecular size, charge or substrate specificity. A more rational system is now based on a comparison of active sites, mechanism of action, and three-dimensional structure (IUB, 1984). A detailed

mechanistic description of a protease can therefore range from a crude classification based on susceptibility to a group of protease inhibitors (e.g. PMSF and PCMB) to an elegant discussion of stereoelectronic factors based on high resolution X-ray crystallography.

Within these extremes are numerous experimental protocols for obtaining data on the mechanistic grouping of a new activity. Among these are response to a broader range of inhibitors, chemical modification studies, pH dependence of activity, solvent deuterium isotope effects, and cleavage site specificity studies. The latter are helpful in designing a more selective inhibitor and allow a further distinction into 'mechanistic' subclasses, such as 'trypsin-like serine protease'. Nevertheless, four mechanistic classes (i.e. serine- (EC# 3.4.21), cysteine- (EC# 3.4.22), aspartic- (EC# 3.4.23), and metallo-proteases EC# 3.4.24) are recognized by the International Union of Biochemistry (IUB, 1984). Six families are established within the classes. Each family has a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site. Members of each family are believed to have descended from a common ancestor by divergent evolution.

2.5 Plant proteases

Cysteine proteases are found in bacteria, (Jocelyn, 1972), in eukaryotic microorganisms, in plants (Glazer and Smith, 1971) and in animals (Barrett, 1986). After studying proteases in the green leaves of eleven plant species, Singh (1962) established that they belonged to the SH class and had a pH optimum between 5.2 and 5.7. Also amino acid sequence data quoted by Barrett (1986) showed that cysteine proteases of other higher plants are members of the papain superfamily. pH response is one of the methods used to elucidate the mechanistic patterns. Using this method, Zucker *et al.*, (1985) showed that papain and related plant cysteine proteases generally show maximal activity on synthetic substrates and proteins at neutral or slightly alkaline pH. Earlier, Ragster and Chrispeels (1981), concurred with Drivdahl and Thimann (1977) who found that proteases in senescing leaves had an active sulphydryl residue. Senescence, like germination (Csoma and Polgar, 1984) is accompanied by protein breakdown and a proportionate increase in free amino acids. Both were inhibited by sulphydryl reagents.

It should be mentioned however that not all the CYS-SH groups in plants are involved in catalysis. Sugiyama *et al.* (1968) found that out of a total of 96 CYS-SH groups per mole of Fraction 1 of spinach protein, approximately 10 CYS-SH groups were blocked before appreciable loss of ribulose 1,5-diphosphate (RuDP) -carboxylase activity occurred. Complete inhibition of enzyme activity was associated with the blocking of about 30 CYS-SH groups. The possible role of CYS-SH groups in structural rigidity of the protein was therefore suggested.

Plant proteases have been associated with some of the changes which occur to the nitrogenous components of ensiled herbage. However, with the proteases responsible having an active cysteine catalytic site, silage preservation strategies should naturally strive to inactivate this highly nucleophilic locus. Ideally, the conservation of a green crop as silage should take place with insignificant loss of nutrients. About 75 to 90% of the total nitrogen in fresh herbage is present as protein, the remaining non-protein nitrogenous fraction consisting mainly of free amino acids and amides (glutamine and asparagine) with smaller concentrations of ureides, amines, nucleotides, chlorophyll, low molecular weight peptides and amino acids bound in non-protein form (Ohshima and McDonald, 1978). The concentration of nitrates present depends upon plant species and manuring. The amount of free ammonia-N in fresh herbage is usually less than 1% of total nitrogen. Furthermore, with ensiling, extensive hydrolysis of water soluble carbohydrates (WSC) and protein occurs, resulting in the production of lactic acid and an increase in soluble non-protein nitrogen (NPN). Depending on the extent of wilting prior to ensiling, up to 60% of the total nitrogen may be degraded to NPN, while lactic acid is converted to butyric acid. Often, this occurs when crops are ensiled directly after harvesting. Unless measures are taken to enhance intake of the ruminant animal, rumen microbial protein synthesis and possibly protein digestibility, the efficiency of nitrogen (N) utilization by ruminants fed silage with such high NPN content is poor (McKersie, 1985).

2.6 Proteases of the rumen

The location and properties of proteolytic enzymes of rumen bacteria have been the subject of investigation by Kopecny and Wallace (1982). Their results indicate that most activity was removable by gentle physical methods such as shaking and brief blending without cell disruption viz., it was associated with coat and capsular materials. Proteases were also present intracellularly and in the cell envelope, corresponding to the inner membrane fraction of gram-negative bacteria. These were removable by detergents. Blending also increased cellulase activity. Detergents (Triton X-100, Nonidet) also increased the total proteolytic activity, possibly due to unmasking of enzyme activity. Triton X-100 is known to extract the inner membrane proteins of gram-negative bacteria (Schnaitman, 1971). From the effects of protease inhibitors however, Wallace and Cotta (1988) concluded that the predominant type of protease present in rumen contents, mixed rumen bacteria and extracted capsular enzymes is a cysteine (CYS-SH) -protease type, sensitive to p-chloro-mercuribenzoate (PCMB, 56-89% inhibition). Other types of activity are also present, but are more variable. These include phenylmethylsulphonyl fluoride (PMSF)- sensitive serine protease (present at 0-41% of total activity), metalloprotease (9-30%) and aspartic protease (2-15%). From the foregoing evidence, the importance of the CYS-SH groups in rumen fermentation was firmly established.

Evidence however, exists that the type of protease present in the rumen is influenced by the animal diet (Prins *et al.*, 1983; Wallace and Brammall, 1985). Concentrate diets were associated with the increased presence of serine-proteases. According to Prins *et al.* (1983), a cow on a hay diet had no serine-proteases in its

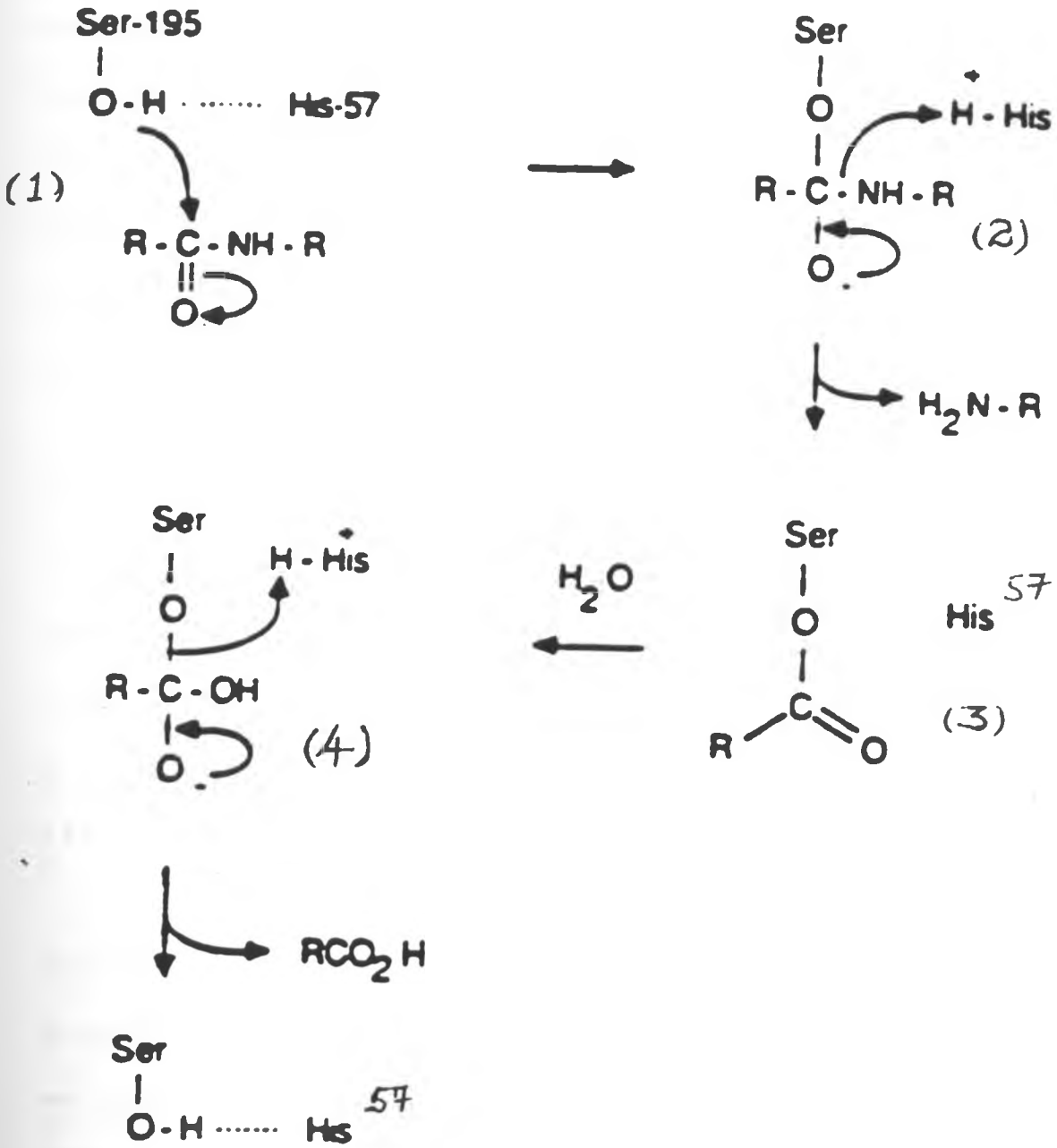
rumen bacterial fraction. Furthermore, it was noted that representatives of most bacterial species have some proteolytic activity, with the possible exception of the main cellulolytic bacteria, Bacteroides succinogenes, Ruminococcus flavefaciens and R. albus (Wallace and Cotta, 1988). Cereal diets yield higher proteolytic activities than do forage diets, probably because rumen microorganisms tend to be amylolytic rather than cellulolytic. The breakdown of amylose may provide energy required by the bacteria. The mechanistic similarity of amylose and protein breakdown is exemplified by Bacteroides amylophilus, one of the most active amylolytic and proteolytic species isolated. Wallace and Brammall (1985) found that this specie has a predominantly serine protease active site. Consideration of the mechanism of serine-protease is therefore warranted.

2.7 Catalytic mechanism of serine proteases

The active site of a typical serine protease is made up of two regions: (a) the catalytic site, and (b) the substrate binding site(s) (Powers and Harper, 1986). As would be expected, most serine protease inhibitors interact with both of these regions.

Powers and Harper (1986) have outlined the following serine mechanism: Ser-195, His-57 and Asp-102 (chymotrypsin numbering system) are the catalytic residues. These three residues form a hydrogen bonding system often referred to as the 'catalytic triad' or in older literature, as the 'charge relay system'. The aspartic residue is normally buried in the structure of the enzyme. Hence, the serine and the histidine residues are the most frequent targets of inhibitors. Fig. 2.1 shows the catalytic pathway. Locations within the mechanism are numbered from (1) to (4).

Fig. 2.1. Catalytic mechanism of a serine protease (Powers and Harper, 1986). *The catalytic residues are serine-195, histidine-57 and aspartic acid-102 (not shown).*



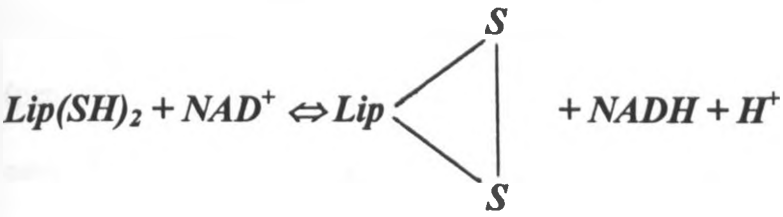
After substrate binding, the unusually reactive α-hydroxyl group of the active site serine (Ser-195) attacks the carbonyl of the scissile amide bond (peptide bond) of the substrate to give a tetrahedral adduct (2). The imidazole side chain of the adjacent histidine residue facilitates this process. Hydrogen bonding to the backbone NH groups of Gly-193 and Ser-195 of the enzyme stabilizes the tetrahedral adduct making the 'oxyanion hole'. The decomposition of the tetrahedral adducts results in the release of the amino portion of the substrate and the formation of an acyl serine derivative (3). The departing amino group receives a proton from the imidazole ring of His-57 (4). Subsequent hydrolysis of the acyl enzyme to active enzyme and the carboxylic acid product occurs through a reaction sequence that is analogous to that involved in formation of the acyl enzyme.

Chymotrypsin and subtilisin are representatives of the two families of serine proteases. In subtilisin, the side chain of Asp-155 is believed to be one of the donors of a hydrogen bond forming the oxyanion hole. It is obvious that the exclusive use of subtilisin for the in vitro estimation of rumen protein degradation omits the predominant CYS-SH protease activity. A combination of subtilisin and papain, trypsin or chymotrypsin would be preferable.

2.8 Role of SH groups in enzymes

Jocelyn (1972) reviewed the functions of SH and SS groups in enzyme catalyzed reactions. From this review, three roles are clearly associated with the SH groups in enzymes namely, catalytic, binding of substrates and cofactors (metal ions and coenzymes), and maintaining the native protein conformation. It is the catalytic role that is of significance to this study.

Under this role SH groups of proteins may act as (a) nucleophilic catalysts (Rich, 1986; Barrett, 1986) or, (b) as intermediate electron carriers of oxidative enzymes from substrates to such acceptors as NAD^+ (Barron, 1951). The latter role is exemplified by lipoamide dehydrogenase (NADH: lipoamide oxidoreductase, E.C. 1.6.4.3). This enzyme is a component of the α-ketoglutarate and pyruvate dehydrogenase complexes and catalyzes the following reaction:



A number of enzymes are known in which the direct participation of covalent acyl ester groups in nucleophilic catalysis is unambiguously proven. Amongst them are, trypsin, chymotrypsin and papain. However, evidence for the formation of covalent acyl-thioester intermediates during the catalytic cycle was first obtained with D-glyceraldehyde 3-phosphate dehydrogenase (GPDH, EC 1.2.1.12) (Krimsky and Racker, 1954, 1955). An enzyme of glycolysis, GPDH plays an important role in the metabolism of carbohydrates in most living organisms including rumen bacteria. In the presence of NAD^+ and phosphate, it catalyzes the reversible oxidative phosphorylation of 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid. In the earliest work devoted to papain, it was found that this enzyme is activated by H_2S and cyanide, and is inhibited by various thiol reagents (iodine, hydrogen peroxide, quinone, iodoacetate, Cu_2O, and the organomercurials) (Klein and Kirsch, 1969 a,b). Klein and Kirsch (1969a) also found that the activity of papain preparations is directly proportional to

the content of free SH groups. Further, they showed that the inactive form of papain is a mixed disulfide formed by the SH group of the protein and cysteine.

2.9 Catalytic mechanism of cysteine proteases

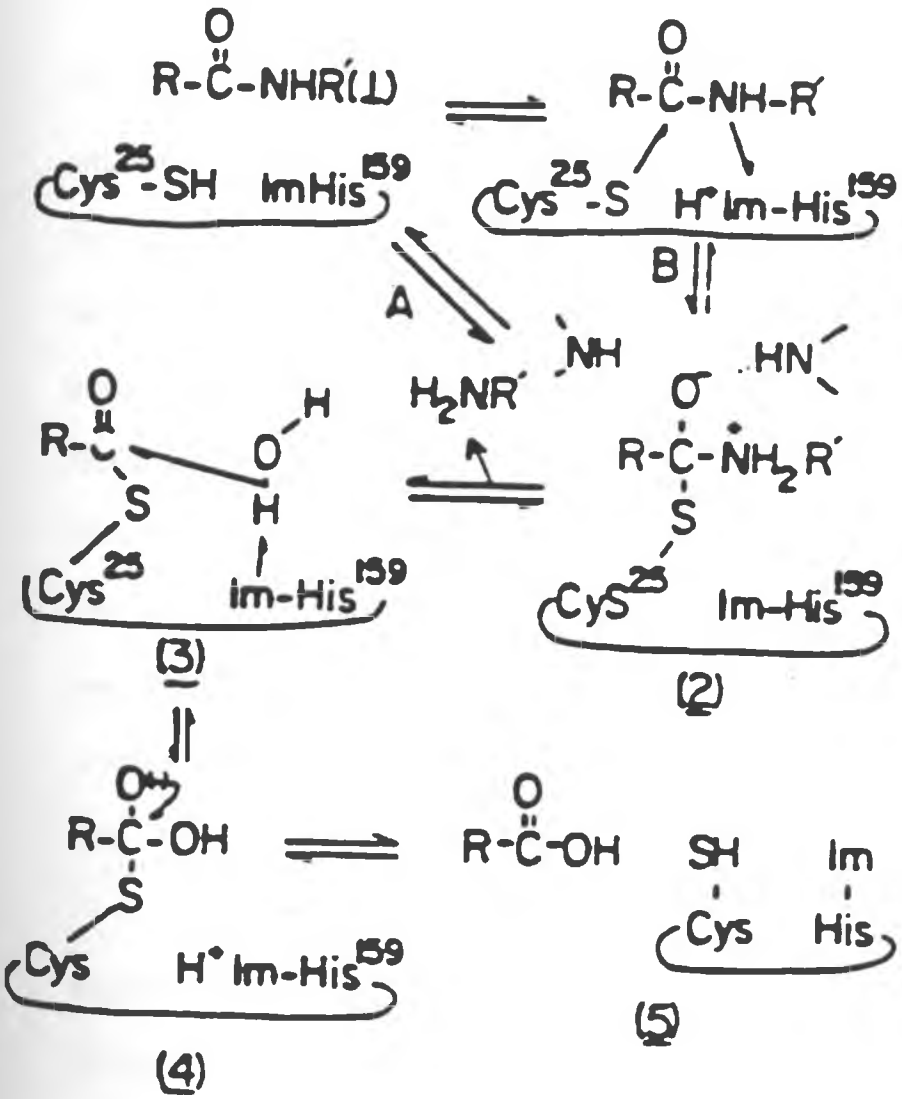
Rich (1986) provided a detailed account of the catalytic mechanism of cysteine proteases (see Fig. 2.2). A catalytically active cysteine sulphydryl group (Cys-25) and a histidine imidazole group (His-159) are contained within the active site of the enzyme. The enzyme functions by the attack of the Cys-25 sulphydryl group on an amide (peptide bond) or ester carbonyl group in (1) to form a covalent intermediate, the thio ester (3) in papain with release of the first product. The minimum reaction mechanism can be represented by the following pathway;

$$E + S \Leftrightarrow ES \Leftrightarrow ES' + P_1 \Rightarrow E + P_2$$

The thio ester (3) (corresponding to ES'), was prepared synthetically by reaction of papain with active esters (Bendall and Lowe, 1976). Upon standing, the acyl group of thio ester is transferred to a water molecule in a relatively slow process to liberate the carboxylic acid (5) and regenerate the active enzyme. The reaction of water on the thio ester in the deacylation step is catalyzed by the imidazole group of His-159.

There is some uncertainty as to where the proton is located in the resting state of the enzyme and during catalysis (Lowe, 1976). However, the imidazole group is understood to polarize and deprotonate the Cys-25 sulphydryl group. The ion pair species depicted in Fig. 2.2, which equilibrates with the neutral form, would be highly nucleophilic. The ionic form permits the imidazolium ion to protonate the nitrogen of .

Fig 2.2. Mechanism of hydrolysis of peptide substrates by cysteine proteases (Rich, 1986)



the leaving group while the Cys-25 thiolate attacks the carbonyl carbon. This mechanism would rationalize the unusual reactivity cysteine proteases show towards electrophiles relative to the nucleophilicity of sulphur in cysteine or glutathione in solution, especially at lower pH values where deprotonation of thiols is less favoured.

2.10 Similarities between serine and cysteine protease

Serine and cysteine proteases form covalent enzyme complexes in contrast to aspartic and metallo-proteases. This distinction into two major mechanistic groups is of profound consequence since the strategy for manipulation is totally different for the two classes. Enzymes that form covalent complexes have strongly nucleophilic amino acids at their catalytic site. These are usually aligned with hydrogen bond acceptors to promote the dissociation of the nucleophile in the approach to the transition state, and thus increase the fraction in the hyper-reactive state. Design and synthesis of inhibitors of this broad class has hitherto concentrated on introduction of electrophilic groups *(-C-C(=O)-Cl, HCHO* etc.) that would chemically modify the nucleophile or general base. This would render the catalytic apparatus inactive and prevent the action of that protease.

2.11 Differential reactivity of SH groups of proteins

SH groups of proteins are usually divided into three types: readily reacting, sluggishly reacting, and "masked" or "buried". According to Barron (1951), SH groups of the first type react easily with nitroprusside and with mild oxidants such as ferricyanide, porphyrindin, and o-iodosobenzoate. The SH groups of the second type do not give a nitroprusside reaction and react only with stronger oxidizing agents (e.g. iodine) and with mercaptide-forming reagents (p-chloromercuribenzoate and organic

arsenic compounds). The third category of SH groups can only be detected after denaturation of the protein. In a completely denatured protein all of the SH groups have a reactivity similar to that of low molecular weight thiols.

Rabin and Whitehead (1962) postulated that SH groups located in the active sites of enzymes are activated by formation of hydrogen bonds with neighbouring functional groups of protein, e.g. with imidazole or with carboxylate anions. The participation of the proton of the SH group in the formation of a hydrogen bond leads to an increase in electron density on the sulfur atom and consequently enhances its nucleophilic properties. Along with this there must be an increase in the pK (dissociation constant) of the SH group and a decrease in the pK of the proton-accepting group (e.g., imidazole), since both ionization of the SH group and protonation of the imidazole involve breaking of the hydrogen bond.

Polgar (1979) suggested that the SH groups of both papain and GPDH are activated as a result of complete transfer of the proton from the SH group to a neighbouring imidazole to form an ion pair. The ion pair presumably predominates over the pH range 4.2 to 8.6. Heitmann (1968) studied the reactivity of SH groups introduced into anionic and cationic micelles. He observed that the insertion of SH groups into anionic micelles is accompanied by their "masking ", i.e. by a sharp decrease in their reactivity. Conversely, when inserted into cationic micelles, SH groups display a sharp increase in reactivity.

"Masking" of SH groups in proteins implies that reactions with various specific thiol reagents are retarded or absent. Two hypothesis have been advanced to explain the mechanism of "masking": a) The SH groups are sterically inaccessible, i.e., they are

spatially screened by neighbouring amino acid residues; b) The SH groups form intramolecular chemical bonds, i.e. they are "chemically masked".

According to the first of these hypothesis, sulphydryl and other functional groups that are characterized by diminished reactivity are located inside the protein molecule. They are, therefore sterically inaccessible to chemical reagents. In support of this view is the long known fact that the reactivity of SH groups increases upon denaturation of proteins, i.e. upon unfolding of the globule.

Thiol groups of proteins may be masked not only by purely ionic interactions but by the formation of various kinds of intramolecular chemical bonds. Thus, Linderstom-Lang and Jacobsen (1941) suggested that the diminished reactivity of some SH groups of proteins could be explained by formation of thiazolidine or thiazoline rings. These workers also suggested the formation of thioester linkages from SH and COOH groups and the formation of hydrophobic interactions as causes of masking in proteins.

2.12 Ionization of thiols

Thiol groups take part in most reactions as mercaptide (thiolate) ions, RS^-. Therefore, to evaluate the reactivity of the SH groups of proteins it is important to know at what pH these groups ionize.

The individual ionization constants of the -SH and $-NH_3^+$ groups of cysteine and related compounds were calculated by Benesch and Benesch (1955) from spectrophotometric titration data. From their data, it follows that -SH and $-NH_3^+$ groups are acids of almost equal strength. However, the SH group of cysteine is a

somewhat stronger acid than is the $-NH_3^+$ group. In addition, the pk_a of the SH group in cysteine is diminished under the influence of the positively charged amino group.

The pk_a values of SH groups of proteins vary within wide limits under the influence of a variety of factors. The spatial arrangement of SH groups within the protein macromolecule is one such factor. The so- called "buried" or "masked" SH groups, arranged in a hydrophobic environment inside the protein and having no contact with the solvent, evidently are characterized by higher pk_a values than are the SH groups on the surface. The pk_a values in the active sites of enzymes vary from 4 to 9. It is highly probable that these groups are located on the surface of the enzyme macromolecules or in clefts on the surface.

An important factor influencing the value of the ionization constants of SH groups in protein molecules is the electrostatic influence of neighbouring charged groups. On the basis of the behaviour of low molecular weight thiols, it may be concluded that the presence of a positively charged group in the immediate vicinity of an SH group lowers its pk_a to about 8.35 (the pk_a of β-mercaptoethylamine); in the absence of nearby charged groups, the pk_a of an SH group may be about 9.9 (as in β-mercaptoethanol); in the presence of a nearby negatively charged group, the pk_a of the SH group of a protein may rise to 10.3 (as in β-mercaptopropionic and mercaptoacetic acids). The heat of ionization of SH groups is about 7 kcal/mol; this corresponds to a decrease of 0.2 in the pk_{SH} for an increase in temperature from 25 to 37 °C (Benesch and Benesch, 1955).

2.13 Mechanism of cellulase enzymes

The term cellulase encompasses a melange of hydrolytic plus some oxidative enzymes, that interactively promote the degradation of cellulose. The classical action of cellulases is envisioned to involve an initial attack by endoglucanases followed by the combined action of cellobiohydrolases, exo- and endo-glucanases, with final hydrolysis of the oligosaccharides to glucose by cellobiase (Goyal, *et al.*, 1991).

Lysozyme-like mechanisms have long been postulated for cellulases, but to date supportive evidence, although intriguing, is quite meagre (Yaguchi, *et al.*, 1983). Site directed modification of cellulases and studies of cellulases in the presence of substrate and/or inhibitor have provided some evidence regarding the involvement of carboxyl groups in the active site. Furthermore, using N-bromosuccinimide as the modifying carbodiimide, it has been deduced (Clarke and Yaguchi, 1985) that two tryptophan residues in the proposed active cleft of Schizophyllum commune endoglucanase are involved in catalysis, one residue binding to the substrate while the other forms part of the active site. Evidence from isolation of a catalytic peptide (Tomme and Claeyssens, 1989) helped to postulate the involvement of glutamic acid 126 (as a proton donor) in the catalytic site of Trichoderma reesei, this being equivalent to glutamic acid 35 of hen egg white lysozyme.

Site directed mutagenic modification of the proposed active site is also being used to resolve which amino acids are directly involved in hydrolysis. According to Goyal *et al.*, (1991), Shoemaker proposed that one of the two aspartyl residues (173 or 175) of T. reesei is active in catalysis, and proved that one of them is involved.

Replacing both of them with asparagine residues resulted in a protein enzyme inactive towards swollen cellulose.

2.14 Manipulation of anaerobic fermentation

In silage preservation, the decrease in pH has been identified as contributory to successful fermentation (McKersie, 1985; Muck, 1988). Low pH reduces the ionization and consequently the reactivity of the plant cysteine proteases. Nonetheless, it takes time through ordinary fermentation to obtain a pH low enough within the intracellular sites of active proteolysis. Hence, attempts have been ongoing aimed at direct acidification (Muck, 1988), and inoculation with either enzymes or lactic acid bacteria (McDonald, 1981; Muck, 1988). Success with these methods has been variable.

In anaerobic reactors, Tween 80 resulted in a significant increase in enzyme efficiency (Castanon and Wilke, 1981; Helle *et al.*, 1993). By monitoring sugar production from cellulose, Castanon and Wilke (1981) found that 0.1% Tween 80, improved the efficiency of enzyme usage by 33%. A number of studies have also reported the ability of surfactants to prevent cellulase inactivation (Reese, 1980; Kim *et al.*, 1982; Helle *et al.*, 1993). These reports have suggested that surfactants provide attachments between the hydrophobic sites of cellulose and the enzymes. Applications of surfactants in silage-making in conjunction with enzymes has not been documented.

In rumen studies, Tween 80 was associated with considerable cell lysis (White *et al.* 1988). However, Akin (1980) reported that 0.1% Tween 80 increased viable bacterial count in some cases by as much as 70%. Similar findings were made by Dehority and Grubb (1980). Hazlewood and Dawson (1979) isolated a naturally occurring fatty acid-requiring Butyrivibrio sp. (strain S2) from the ovine rumen that

deacylates plant galactolipids, phospholipids and sulpholipids to obtain sufficient fatty acid for growth. Growth in vitro was promoted by adding to the growth medium straight-chain saturated fatty acids (C_{13} to C_{18}). On testing Tweens 20, 40, 60 and 80, it was found that all supported similar growth. Rothfield and Finkelstein (1968), Triggle (1970), and Machtiger and Fox (1973) reviewed evidence that shows cell and bacterial membrane associated enzymes could be activated by nonionic surfactants. The mechanism of action seems unclear. However, Triggle (1970) has suggested that surfactant lipids may provide sites for enzyme-substrate hydrophobic interaction. Since the catalytically active SH groups of most cysteine- proteases are located in hydrophobic environments within the enzyme protein molecules, nonionic surfactants would enhance substrate binding.

Inhibition of protein and amino acid degradation to ammonia is one of the main aims of improving nitrogen use by the ruminant (Jouany, 1994). Among the compounds suggested are diaryliodonium salts and hydrazine-types (Broderick *et al.*, 1991). These products have inhibitory effects on the degradation of rumen amino acids and methanogenesis. However, their high toxicity excludes their use in animal feeds. It should be noted that although diaryliodonium salts, fluorodinitrobenzene (FDNB), chlorodinitrobenzene (CDNB), and trinitrobenzenesulfonic acid (TNBS) are usually regarded as reagents for protein amino groups, as a chemical rule they combine even more rapidly with SH groups (Wallenfels and Streffer, 1966). Others, e.g. 4-nitrobenzofurazans and 4-nitrobenzofuroxans are considered thiol-neutralizing agents (Whitehouse and Ghosh, 1968).

According to Jouany (1994) these compounds limit the degradation of amino acids by inhibiting their transport through the bacterial cell wall. This is not surprising in that microbial membranes are high in SH content (Jocelyn, 1972; Levitt, 1980). These authors also noted that SH reagents reduced membrane permeability including active transport. Similarly, Jocelyn (1972) indicated that the capacity of rumen bacteria to synthesize methane is inhibited by mercurials. That Hino and Russell (1985) observed a decrease in both the NAD^+/NADH ratio and deamination of amino acids when methane production was inhibited concurs with the foregoing. As noted earlier, SH groups of oxidative enzymes act as intermediate electron carriers from substrates to such acceptors as NAD^+: Inactivation of the intermediate SH electron carrier groups would cause an accumulation of NAD^+ consequently lowering the above mentioned ratio. According to Van Nevel and Demeyer (1988), Ag^+, Cd^{2+}, Cu^{2+} and Hg^{2+} ions were also effective in inhibiting proteolysis probably due to their interaction with SH-enzymes. Lastly, it should be mentioned that the effects of protease inhibitors hitherto proposed on the integrated system of ruminal carbohydrate fermentation, microbial growth and lower tract digestion are largely unknown and merit further research.

2.15 Effects of surfactants on thiol reactivity

The most well known physicochemical property of surfactants is their interfacial activity - the surfactant molecules migrate to the interface(s) when placed in solution. Surfactant molecules actually align at the interface(s) by virtue of their "amphipathicity". Their alignment at interfaces reflects the tendency to assume the most energetically stable orientation. Surfactants are also commonly classified on the basis of (a) the charge and/or nature of the hydrophilic portion (head), and (b) the

flexibility and/or chemical nature of the hydrophobic portion. Head groups may be anionic (e.g., SDS, Teepol HB7), zwitterionic, nonionic (e.g., Tween 20, 40, 60, and 80) or cationic (e.g., Cetyltrimethylammonium bromide -CETAB). The most common surfactants encountered in foods have been reviewed by Griffin and Lynch (1972) and Artz (1990). In other biochemical applications, Neugebauer (1988, 1990) gave excellent reviews. Strikingly missing from these reviews is the effect of low levels of surfactants on enzyme activity and in particular thiol proteases and cellulases.

The fact that surfactants affect the properties of food proteins was discussed by Sasago *et al.*, (1963) and Nakai and Li-Chan, (1988). Working with milk proteins the former observed a decline in the reactive SH content in the presence of 0.25% SDS. Nakai and Li-Chan, (1988) discussed the effects of covalent attachment of fatty acids, fatty acid derivatives, or surfactant molecules to proteins, in the process called lipophilization. Increased hydrophobicity of proteins with consequent improvement in the relative affinity for nonpolar compounds was noted. Noncovalent attachment or binding of low levels of anionic detergents to proteins was observed to increase the protein's thermal stability and dispersibility (solubilization). Heitmann (1968) investigated the reactivity of protein thiol groups in the presence of anionic (sodium N-dodecanoyl-DL-cysteinate), nonionic (Brij 35) and cationic (CETAB) surfactants. His results showed that anionic micelles significantly reduced SH reactivity, while CETAB accelerated their reactions by three orders of magnitude. The effect of Brij 35 was positive but very small.

Polyoxyethylene sorbitan esters are synthesized by the addition, via polymerization, of ethylene oxide to sorbitan fatty acid esters. These nonionic

hydrophilic emulsifiers are very effective antistaling agents and are therefore used in a wide variety of bakery products (Artz, 1990). They are much more widely known as polysorbates (see Figure 2.3).

The chemical structure of polysorbate 80 (Tween 80) is given as;

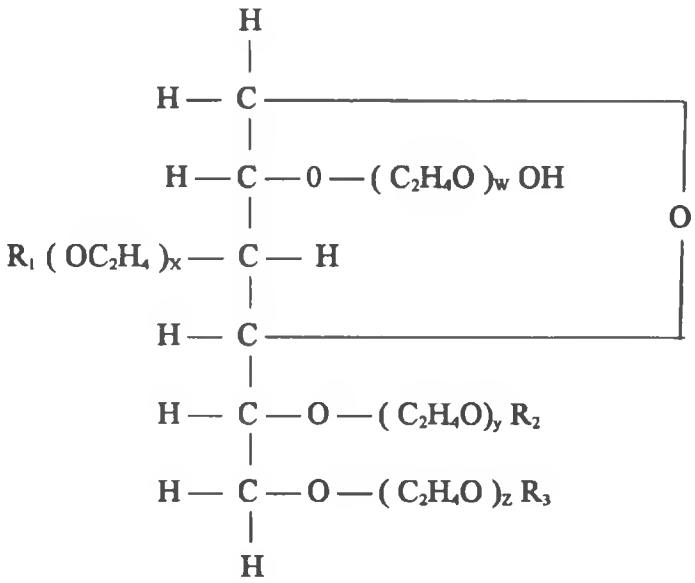


Figure 2.3. Polysorbates, *where w + x + y + z = 20 (approximately) and the R's represent a single oleic fatty acid and hydrogen.*

It is evident therefore that surfactants could play a significant role as feed additives particularly in the control of protein fermentation. As observed by Castanon and Wilke (1981), and Helle *et al.* (1993) Tween 80 could be used to enhance cellulose breakdown. Further, Heitmann (1968) noted that CETAB increased protease activity. Either of these surfactants or their combination could be used to enhance forage degradation in the rumen. In contrast, anionic surfactants, e.g. Teepol or SDS could be used to enhance CYS-SH masking, thus reducing their protease activities. Consequently, this provides an avenue for the inhibition of proteolysis both in the silo and in the rumen. Inactivation of catalytic SH groups could also be achieved through

the use of mild oxidants, e.g. KIO. CYS-SH groups are known to be very susceptible to oxidation. In baking powder, KIO is used to oxidize the CYS-SH groups in wheat gluten.

Hence, it was proposed that the application of low levels of anionic surfactants be investigated in attempts to enhance ruminal protein bypass and suppression of silage proteolysis. Experiments were also designed to incorporate a nonionic surfactant (Tween 80) in a forage based ration for evaluation of digestibility. In addition, in vitro studies on the effects of low levels of these surfactants on the reactivity of CYS-SH groups of proteases was proposed. Two surfactants namely, Tween 80 (polyoxyethylene monooleate -nonionic) and Teepol HB7 (Sodium alkyl sulfate - anionic) were selected for testing on the basis of low cost. Tween 60 and SDS were also used in some comparisons.

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Chapter 3

ENHANCEMENT OF FORAGE FERMENTATION IN THE RUMEN

3.0 ABSTRACT

This study was designed to investigate the effects of Tween 80 on the activities of rumen proteases and cellulases and on in vivo feed intake and nutrient digestibility in sheep. Proteases extracted from mixed rumen microorganisms were concentrated on an Amicon XM-300 filter and used in an in vitro test designed to determine the effect of Tweens 60 and 80 on protease activity, SH reactivity, and the apparent microbial enzyme protein adsorption to a barley straw substrate. The Km value for apparent protease activation for Tweens 60 and 80 was shown to be 0.28 and 0.20%, while the Vmax values were 99.2 and 166.8 (%/unit additive conc), respectively. Compared to the control there was significant SH unmasking particularly with Tween 80. At 0.25 % in the final reaction mixture, the initial rates of cellulose degradation for the control, Tweens 60 and 80 were 0.60, 0.87, and 1.04 (μg/ml/h), respectively. Apparent microbial enzyme protein adsorption to finely ground barley straw increased significantly with concentrations up to 0.25 % Tween 80. The addition of 0.5% Tween 80 to a grass hay ration increased DMI (as % BW) by 40 % and nutrient digestibility (DM, ADF, NDF, and CP) by approximately 10 percentage units in a feeding trial carried out with sheep. These results indicate the potential use of Tween 80 in ruminant rations.

(**Keywords**: rumen, surfactant, digestibility, Tween 80)

3.1 INTRODUCTION

Ruminant animal production remains dependent on forages as the major feed source. It is believed that the efficiency of feed utilization by ruminants has remained unchanged during the last two decades (Cheng *et al.*, 1991). Since feed is a major cost in ruminant production, enhancing digestive efficiency will remain a primary objective in the industry. Consequently, an in-depth understanding of the roles of feed processing and microbial digestion is required to fully manipulate the digestive processes in the rumen. The importance of the microbial enzyme-substrate complex in the digestion of feeds is well known. Feed processing practices, e.g. grinding, endeavor to increase microbial enzyme-substrate interaction by exposing susceptible substrate binding sites. However, progress remains to be made in controlling microbial enzyme adsorption and the subsequent catalysis. The success of this strategy may hinge on the progress made in similar fermentation processes.

The rumen microbial ecosystem is complex, with many microbes and reactions, but it is not unique. Similar ecosystems exist in anaerobic digesters and sanitary landfills. In anaerobic digesters, conventional practices have been coupled to microbial enzyme activation using surfactants with remarkable increases in production efficiency. Using as little as 0.1 % Tween 80, a nonionic surfactant, Castanon and Wilke (1981) and Helle *et al.* (1993), increased the rate and extent of cellulose saccharification by almost 40%. The enhanced activity was believed to be mediated by increased cellulase stabilization and attachment to the substrate binding sites and possibly increased

unmasking of catalytic binding centres, thus enhancing enzyme access. A decade ago, tests with rumen fluid showed increased viable bacterial counts of fiber digesting bacteria and enhanced cellulolytic activity in the presence of Tween 80 (Akin, 1980; Dehority and Grubb, 1980). However, no attempts have been made to tailor the use of Tween 80 to enhance the digestion processes of the rumen, even though Tween 80 has wide applications in foods due to its safety (Griffin and Lynch, 1972).

Forage utilization is normally limited by slow rates of rumen fermentation. The potential of enhancing ruminal forage fermentation with Tween 80 could be promising. Hence, the objective of this study was to assess the effect of Tween 80 on rumen protease and cellulase activity, and in vivo forage nutrient digestibility.

3.2 MATERIALS AND METHODS

3.21 Experiment 1. Determination of the effects of Tween 80 on protease activity, thiol reactivity and apparent microbial enzyme protein adsorption

3.211 Animals, feed and rumen fluid collection

Two rumen-fistulated, non lactating Holstein cows (averaging 623 ± 12.5 kg) in weight were each offered 5 kg of medium quality timothy hay plus 2 kg of concentrates twice daily. Ruminal fluid (2.5 L) was collected from each cow through the fistula 4 h after the morning feeding and homogenized using a Polytron (Brinkmann Instruments, Rexdale, Ontario). The coarse feed particles were removed by sieving the fluid through a 0.5 mm strainer followed by centrifugation at 500 gmax (for 10 min). The supernatant obtained was centrifuged at 28,000 gmax to precipitate the bacteria (microbial pellet) for use in the preparation of a microbial enzyme protein powder.

3.212 Preparation of microbial enzyme protein powder

The microbial enzyme protein powder was prepared using the acetone-butanol extraction procedure outlined by Mahadevan *et al.* (1987). About 300 - 500 g of this powder were prepared and stored at - 20 °C. Extraction of the proteases was accomplished by stirring 250 g of the powder with 1 L of 4 °C cold water for 1 h and then by following Mahadevan's extraction procedure. Extracts from the filtration with an XM-300 Amicon Filter, (approx. 300,000 molecular weight cut off - under nitrogen gas), were made, washed twice with distilled water and the retentate freeze dried (lyophilized). This was referred to as the mixed microbial enzyme protein since it

consisted of mixed ruminal enzymes. It was used in the protease activity, protein adsorption tests and also in the thiol reactivity determinations.

3.213 Determination of protease activity and thiol reactivity

Protease activity and thiol reactivity of enzyme proteins were assessed in the presence of graded levels of Tweens 60 and 80 (Laboratory grade, Sigma Chemical Co., St. Louis, MO. USA). Mixed microbial enzyme protein (10 g) was dissolved in 100 ml of warm (37 °C) 0.1 M phosphate buffer pH 6.8 and used as an enzyme inoculant. The final reaction mixture contained 1 ml of the microbial enzyme protein solution, 1 ml of 2 % casein solution in 0.1 M phosphate buffer and 2 ml of the relevant surfactant or its blank (an equivalent amount of the buffer). Ten levels of the two surfactants (Tween 60 and 80) were made, (with 0.1 M phosphate buffer), to provide the following test concentrations in the final reaction mixture, viz. 0, 0.08, 0.16, 0.24, 0.32, 0.4, 0.8, 1.2, 1.6, and, 2.0%.

All incubations were performed in triplicate in 50 ml plastic centrifuge tubes, at 37 °C under a stream of carbon dioxide gas. After 1.5 h, 2 ml of the assay mixture was pipetted out for the determination of reactive thiol (CYS-SH) content (Sasago *et al.*, 1963) and true protein (Smith et al., 1985). At the end of 2 h incubation, the reaction was stopped with 1 ml of 15 % (wt/vol) TCA (trichloroacetic acid), cooled to 4 °C in an icebath, and centrifuged at 10,000 gmax for 10 min. The amino acids and ammonia in the supernatant were assayed using the ninhydrin method (Rosen, 1957). Proteolytic activation (%) was calculated as the increase in amino acids (AA) released over and above the control (glycine equivalent) per h expressed as a percent of the AA released

consisted of mixed ruminal enzymes. It was used in the protease activity, protein adsorption tests and also in the thiol reactivity determinations.

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All incubations were performed in triplicate in 50 ml plastic centrifuge tubes, at 37 °C under a stream of carbon dioxide gas. After 1.5 h, 2 ml of the assay mixture was pipetted out for the determination of reactive thiol (CYS-SH) content (Sasago *et al.*, 1963) and true protein (Smith et al., 1985). At the end of 2 h incubation, the reaction was stopped with 1 ml of 15 % (wt/vol) TCA (trichloroacetic acid), cooled to 4 °C in an icebath, and centrifuged at 10,000 gmax for 10 min. The amino acids and ammonia in the supernatant were assayed using the ninhydrin method (Rosen, 1957). Proteolytic activation (%) was calculated as the increase in amino acids (AA) released over and above the control (glycine equivalent) per h expressed as a percent of the AA released

by the control incubation over the same experimental duration. Hence, proteolytic activation of the control treatment was zero percent. The optimal surfactant inclusion level was calculated by regression analysis (protease activition regressed against the surfactant concentration) and by direct linear plots (% protease activation versus surfactant concentration) (Henderson, 1992). Therefore, Km and Vmax values were obtained.

Reagents for CYS-SH were prepared as indicated by Sasago et al. (1963). However, the corresponding level of Tween 80 was again added to reestablish the surfactant concentration in the final reaction mixture. Hence, the following solutions were used in the -SH determination. -SH was calculated from the value of optical

No.	*digesta (ml)*	*PCMB (ml)*	*Tween 80 (ml)*	*Water (ml)*	
1	-	-	0.5	0.50	(Blank for No. 4)
2	0.25	-	0.5	0.25	(Blank for No. 3)
3	0.25	0.25	0.5	-	
4	-	0.25	0.5	0.25	

density (OD) at 620 mμ. OD = (OD1 - OD4) - (OD2 - OD3) using a UV spectrophotometer. In the reaction media p-chloromercuribenzoate (PCMB) and dithizone are used. The standard curve for their reaction was prepared by adding graded amounts of PCMB to dithizone and measuring the decrease in optical density (Sasago *et al.*, 1963). The increase in estimated reactive -SH content due to increases in the surfactant concentration was referred to as unmasking -SH reactivity. The rate of unmasking reactive -SH groups was calculated by regressing the reactive -SH content against the surfactant concentration.

3.214 Determination of apparent microbial protein adsorption

The effect of Tween 80 on apparent microbial protein adsorption was studied by suspending a cellulose substrate, (barley straw, with 4 % CP, ground through 0.5 mm sieve), in a solution of microbial enzyme protein. A mother suspension was made containing 4.0 g of lyophilized mixed bacterial enzyme protein in 400 ml of 0.1 M phosphate buffer pH 6.8, with each of the following levels of surfactant Tween-80; 0, 0.05, 0.1, 0.25, and 0.5 %. Apparent microbial protein adsorption was estimated by stirring, (at 120 strokes/min at 37 °C), 0.1 g of barley straw in 5 ml of the microbial enzyme protein solution, and then following the supernatant protein concentration change with time. The following stirring periods were used; 0, 10, 20, 30, 60, and 120 min. All the determinations were carried out in triplicate and replicated three times. At the end of the stirring period, 1 ml of the assay mixture was pipetted into a 5-ml test tube and centrifuged at 2500 gmax for 10 min to precipitate the solids. The protein in the decanted supernatant was precipitated by 15 % (wt/vol) TCA. The precipitate was then freeze dried and true protein quantified by the bicinchoninic acid method (Smith *et al.*, 1985). The rates of apparent protein adsorption were calculated by regressing the adsorbed microbial enzyme protein against time. The extent of microbial enzyme protein adsorption was calculated at 120 min stirring period.

3.22 Experiment 2. Determination of cellulose degradation.

A bacterial fraction, for testing the effect of Tweens 60 and 80 on cellulose degradation, was prepared according to the Forsberg (1978) procedure. This involved centrifuging rumen fluid (digesta) as in experiment 1 above. However, the digesta pellet

was washed, (with 0.1 M phosphate buffer, pH 6.8), and resuspended with an equivalent amount of 0.1 M phosphate buffer pH 6.8 and the rumen fluid supernatant discarded. The resuspended digesta provided both the substrate and the enzyme used in the cellulose degradation assay. The reaction mixture contained 2 ml of resuspended rumen digesta, the test surfactant or 2 ml of phosphate buffer (blank). The concentration of both Tween 60 and 80 in phosphate buffer were adjusted to accommodate dilution, thus providing 0.25% (vol/vol) in the final reaction mixture. This level was based on the Km value obtained from the protease activation test in Experiment 1. Incubations were carried out at 37 °C for periods of 0, 1.5, 3, 6, 12, 24, and 48 h in 50 ml centrifuge tubes. All incubations were carried out in triplicate and replicated three times under a stream of carbon dioxide. The initial and final concentrations of cellulose were determined by the method of Updergraff (1969). Microgranular cellulose was used at graded levels in the preparation of a standard curve. The initial rates of cellulose degradation were calculated by regression analyses.

3.23 Experiment 3. Determination of feed intake and digestibility.

The effect of Tween 80 on feed DM intake and digestibility was investigated in an experiment with four ewes ranging in weight from 49 to 87 kg (averaging 72.5 kg) fitted with a rumen fistulae and a duodenal T-cannulae. The sheep were adapted to the feed in individual pens, and then moved into the digestibility cages during the total collection period. Each sheep was offered 3 kg of grass hay (in two equal portions at 08:30 and 20:30 h) which was either sprayed with 500 ml water or 30 ml of Tween 80 dissolved in 470 ml of water. Water was available ad libitum. The experiment was

designed as a 2x2 latin square with two diets compared in a trial consisting of a 14-day adaptation period and a 7-day collection period and 2 sheep per diet.

During the collection period, records of daily feed intake, and faecal output were maintained. Subsamples (250 g) of the feed, orts, and faeces were collected daily and further subsampled (50 g) for DM determination in an air draft oven at 80 °C to constant weight. Acid and neutral detergent fibre (Goering and Van Soest, 1970), and total N (Parkinson and Allen, 1975) were analysed after grinding the samples through a 0.5 mm sieve.

3.24 Statistical analyses

The data were analyzed using PROC GLM of the SAS Statistical Package (1985). The means were compared using Tukey's Test, and the level of significance was set at ($P < .05$). The rates of unmasking the reactive -SH, microbial enzyme protein adsorption, and cellulose degradation were estimated using regression analysis.

The statistical model employed in the analyses of protease activation, -SH reactivity, microbial enzyme protein adsorption and cellulose degradation data was;

$$Y_{ij} = \mu + P_i + L(P)_{ij} + e_{ij}$$

where, μ = overall mean,

P = period (incubation time for protease activity & reactive -SH) effect

L = additive concentration (levels)

L(P) = levels within periods

e = random error.

For the 2x2 Latin square used in the intake and digestibility trial, the following statistical model was employed;

$$X_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + e_{ijk}$$

where, μ = overall mean

α = treatment effect

β = row effect (experimental period)

γ = column effect (animal group)

e = random error.

3.3 RESULTS

3.31 Experiment 1. Protease activity and thiol reactivity

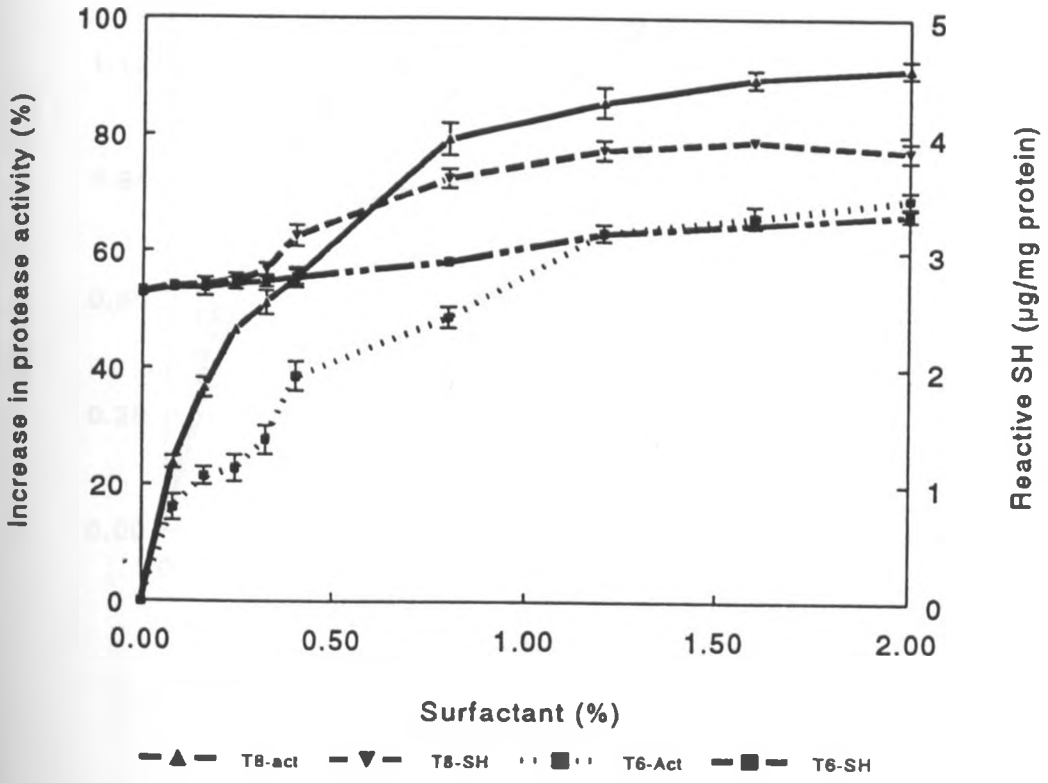
The effects of Tween 60 and 80 on protease activation (increase in activity above the control treatment) of rumen microbial proteases and the increase in thiol reactivity content with change in surfactant concentration (unmasking reactive -SH) are illustrated in Figure 3.1. The rates of protease activation with change in surfactant concentration calculated from regression analyses were, 163.5 ± 4.69 and 98.04 ± 0.13 (%/concentration unit of the surfactant) with Tween 80 and 60, respectively. The optimal surfactant inclusion level was calculated by direct linear plots. The concentration of additive required for mixed rumen microbial enzyme protein to achieve half the maximum velocity (Km value), with the casein substrate is given in Table 3.1. The Vmax value represents the maximum velocity of the enzyme

Table 3.1. The effect of Tween 60 and 80 on the apparent coefficients of protease activation and SH unmasking.

	Protease activation		Unmasking of SH
	Km[1]	Vmax[2]	rate[3]
Tween 60	0.28a	99.2a	0.30a
Tween 80	0.20b	166.8b	0.98b
S.E.M.	0.02	0.57	0.01

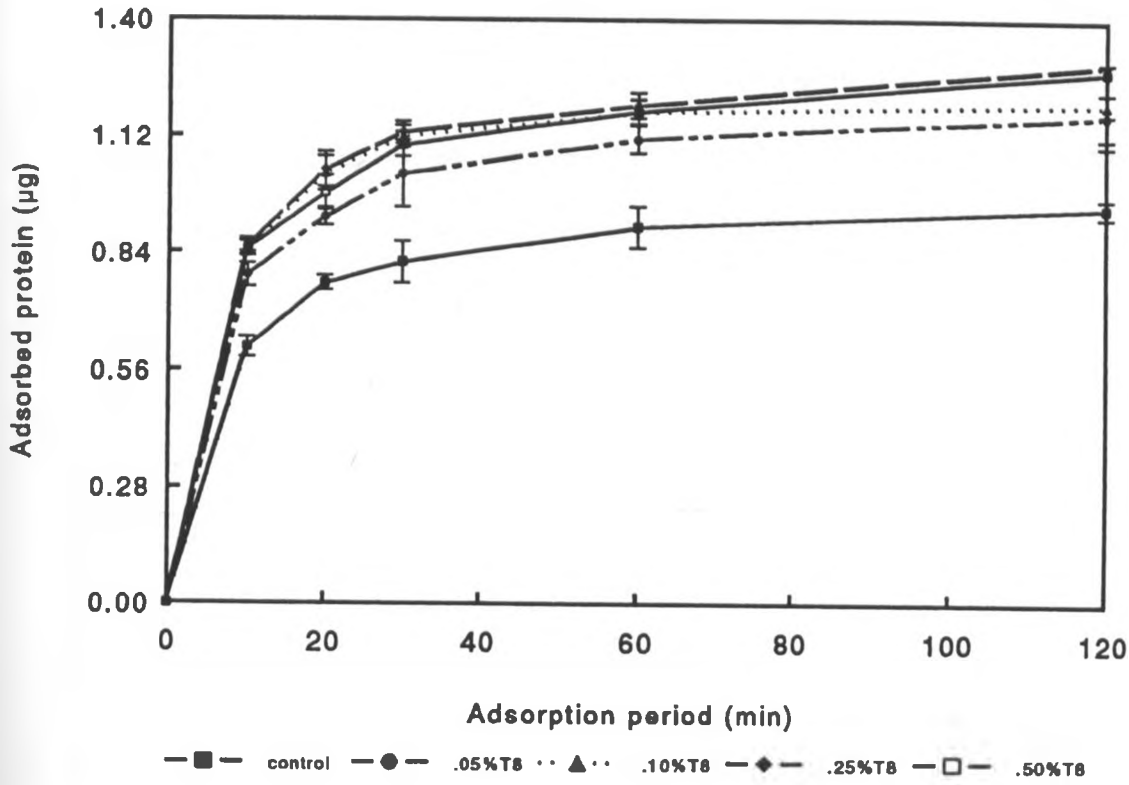
Values within a column followed by different letters differ (P < .05). Standard errors of means obtained from three replicates n=3. [1]Additive conc. (%). [2]Maximum velocity in protease activation (% at (2xKm) conc.). [3]µmol SH/mg protein/unit change in surfactant conc. (%).

reaction - when the casein substrate is at saturating concentration. The velocity of casein degradation (Vmax) in the presence of Tween 80 was higher (P <



Act = Increase in protease activity above the control; T8 = Tween 80, T6 = Tween 60

Fig 3.1 Effect of Tween 60 and 80 on proteinase activity and thiol reactivity



Abbreviations: T8 = Tween 80

Fig 3.2 Effect of Tween 80 on apparent microbial enzyme protein adsorption to barley straw

.05) than in the presence of Tween 60 (Table 3.1). Furthermore, the concentration (Km) of Tween 80 required to elucidate half this velocity was also significantly (P<.05) lower than that of Tween 60.

The rate of unmasking -SH reactivity was higher (P < .05) in the presence of Tween 80 than in the presence of Tween 60 (Table 3.1).

3.312 Apparent microbial enzyme protein adsorption

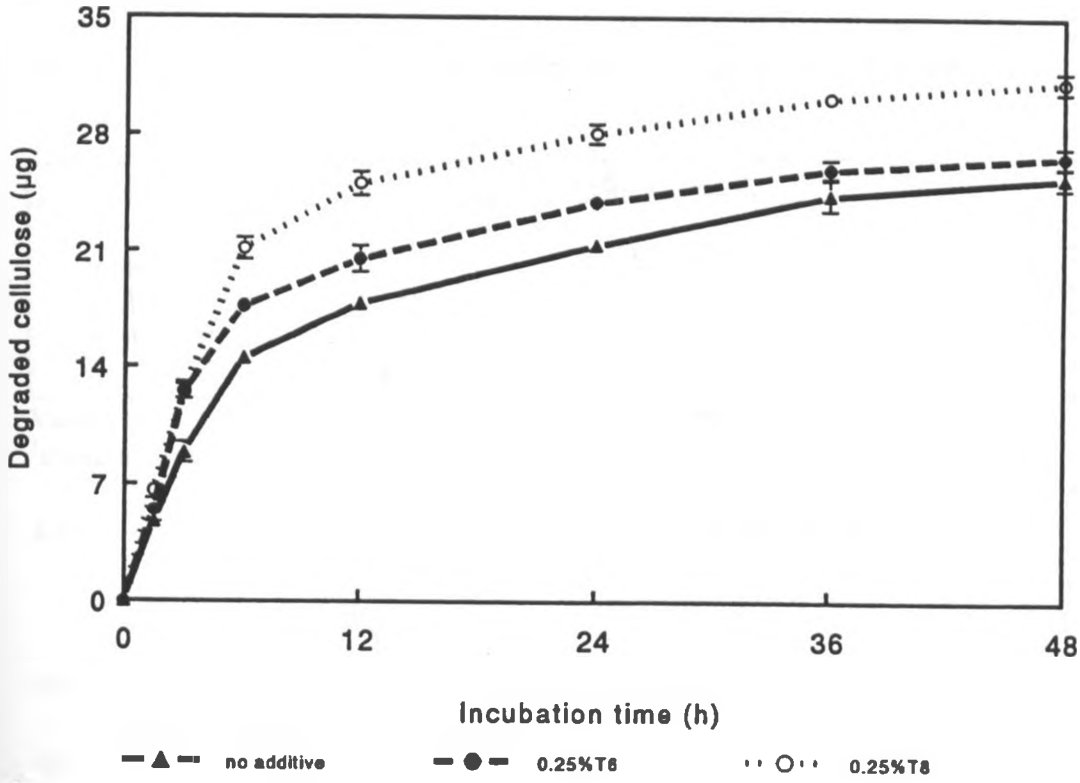
Figure 3.2 shows the effect of Tween 80 on the rate and extent of apparent microbial enzyme protein adsorption to barley straw during 120 min of incubation. The profile of microbial enzyme protein adsorption at 0.1% Tween 80 was very similar to that obtained with 0.25% Tween 80. The addition of Tween 80 significantly (P < .05) increased the apparent microbial protein adsorption to barley straw over that of the control. However, at 0.1, 0.25 and 0.5% Tween 80 in the final reaction mixture

Table 3.2. The coefficients of microbial protein adsorption to barley straw.

Additive level	Microbial protein adsorption			
	rate[1]		extent[2]	
no additive	0.026a	(0.03)	0.94a	(0.01)
0.05 % Tween 80	0.032b	(0.01)	1.12b	(0.03)
0.10 % Tween 80	0.034c	(0.02)	1.21c	(0.03)
0.25 % Tween 80	0.035c	(0.01)	1.18c	(0.02)
0.50 % Tween 80	0.034c	(0.02)	1.19c	(0.03)

Values within column followed by different letters differ (P < .05). Bracketed values are standard errors. n=9. Units: [1] (10^{-3}µg/mg/min); [2](µg after 120 min of incubation).

Neither the rate, nor the extent of apparent microbial enzyme protein adsorption was significantly (P < .05) affected (Table 3.2). The effect of Tween 60 on microbial protein adsorption to ground straw was not determined.



Abbreviations: T8 = Tween 80; T6 = Tween 60

Fig 3.3 Effect of Tween 60 and 80 on in vitro cellulose degradation

3.*313* Experiment 2. Determination of degradation of cellulose

The effects of both Tween 60 and 80 on rumen cellulase activity is depicted in Figure 3.3. The concentration (0.25 %) of both surfactant used in the reaction mixture was based on the approximate Km values (Table 3.1). The rates of cellulose breakdown calculated by multiple regression analysis of the values for the initial 24 h incubation period are shown in Table 3.3. The results show that the addition of either surfactant increased the rate of cellulose breakdown ($P < .05$).

Table 3.3. The effect of Tween 80 and 60 on the initial rates of cellulose degradation.

Treatment	rate (µg/ml/h)	[1]s.d.
no additive	0.60a	(0.21)
0.25 % Tween 60	0.87b	(0.28)
0.25 % Tween 80	1.04c	(0.32)

Values within the column followed by different letters differ ($P < .05$).
[1]standard deviation. n=3. Rates based on the initial 24 h.

3.32 Experiment 3. Determination of feed DM intake and digestibility

On the basis that the in vitro results demonstrated that Tweens 60 and 80 increased cellulose and protein degradation and had a potential for enhanced rumen digestibility of forages, an in vivo trial was carried out. Table 3.4 shows the composition of the grass hay fed to the sheep used in the digestibility trial. Tween 80 was sprayed on the grass hay at 0.5% (vol/wt). Table 3.5 shows the intake and digestibility coefficients for the two rations used in the in vivo trial.

Table 3.4. The chemical composition of the grass hay ration

	Composition				
Feedstuff	**%DM**	**%CP**	**%NDF**	**%ADF**	**%ASH**
Grass hay	82.21 (2.3)	11.8 (1.4)	68.3 (2.1)	33.8 (1.2)	3.4 (0.6)

Bracketed values are standard deviations of the respective means. n=3

The 0.5 % level of Tween 80 tested was approximately twice the Km value, viz., the concentration that elicited maximum substrate protein fermentation in vitro.

Table 3.5. Effects of Tween 80 on feed intake and digestibility coefficients from the sheep trial.

	Control	**Tween 80**	**S.E.M.**
Dry matter intake[1]	1.89a	2.60b	(0.18)
Apparent digestibility (%);			
Dry matter	55.60a	64.70b	(0.49)
Crude protein	52.28a	61.25b	(0.57)
Acid detergent fibre	45.20a	52.68b	(0.43)
Neutral detergent fibre	50.28a	60.63b	(0.61)

[1]expressed as a % of body weight. Values in a row followed by different letters differ ($P < .05$). n=4. Bracketed values are standard errors of means.

Addition of Tween 80 to the diet increased ($P < .05$) both dry matter intake (DMI) and apparent nutrient digestibility. Feed intake was increased by approximately 40% in the presence of Tween 80. The digestibility of feed nutrients was increased by about 10 percentage units.

3.4 DISCUSSION

From Figure 3.1, it is clear that Tween 60 and 80 increased rumen bacterial protease activity ($P < .05$). Of the three proteolytic mechanisms associated with ruminal function, cysteine proteases have the predominant activity (Brock *et al.*, 1982). It is worth noting that most cysteine proteases participate in catalysis as either mercaptide (thiolate) RS^- ions (Rich, 1986), or free thiols (Klein and Kirsch, 1969 a,b). Amongst the factors that could influence the reactivity of these groups is steric hindrance. The SH groups could be unmasked without denaturation by the solubilizing properties of nonionic surfactants (Scopes, 1987). Schnaitman (1971) increased the total proteolytic activity by using Triton X-100 and Nonidet, two nonionic surfactants, during the isolation of rumen proteases. In comparison to Tween 60, Tween 80 would have a higher solubilizing capacity as a result of its slightly higher HLB (hydrophile-lipophile balance). The HLB for Tween 60 and Tween 80 are 14.9 and 15, respectively (Griffin and Lynch, 1972).

It is tempting to attribute the gains in proteolysis wholly to increased enzyme access resulting from both SH unmasking and increased substrate solubility. According to Scopes (1987), higher levels of surfactants (> 0.5 %) would be required to achieve this. Also significant increases in proteolytic activity were observed at low (0.05 - 0.3 %) concentrations of surfactant in the present study. The rate and extent of SH unmasking were not significantly increased at these concentrations. Hence, besides solubility mediated SH unmasking, a different mechanism of activation must be involved, particularly at low surfactant levels. The activation of cell and bacterial

membrane associated enzymes by nonionic surfactants has been widely documented (Rothfield and Finkelstein, 1968; Triggle 1970; Machtiger and Fox, 1973). Although the mechanism of action seems unclear, it was suggested that as a result of their amphipathicity, surfactant lipids would avail sites for enzyme-substrate hydrophobic interaction (Triggle, 1970). Since the SH groups of most cysteine proteases are located in hydrophobic environments within the enzyme molecule, nonionic surfactants could further enhance interaction with potential substrates.

The apparent Michaelis-Menten coefficients in Table 3.1 showed that for purposes of enhancing rumen protease function, Tween 80 would be preferred to Tween 60. Further, to obtain maximum velocity in the proteolytic activity of the mixed microbial enzyme, less Tween 80 would be needed than Tween 60 as shown by a lower Km value for the former additive. Obviously, in vivo benefits would only be made if increased protease activity is coupled to significant increases in fibre fermentation and ultimately to enhanced nutrient digestibility.

Table 3.3 shows that both additives enhanced the initial in vitro cellulose degradation rate compared to the control treatment. However, the effect due to Tween 80 was significantly higher than that due to Tween 60. Nonionic surfactants are used in industrial bioreactors, to enhance cellulose hydrolysis (Castanon and Wilke, 1981; Ooshima *et al.*, 1986; Helle *et al.*, 1993). By monitoring sugar production, Castanon and Wilke, (1981) found that 0.1 % Tween 80 increased the rate and extent of cellulose saccharification and consequently improved the rate of reactor hydrolysis by 33 %. The positive effect due to surfactants was attributed to the prevention of surface

deactivation (Reese, 1980; Kim *et al.*, 1982; Womack *et al.*, 1983), and increased hydrophobic interaction (Helle *et al.*, 1993). White *et al.* (1988) also found a 10 % increase in the activity of exoglucanase A from Ruminococcus flavefaciens with 0.1 % Tween 80. Dehority and Grubb (1980) and Akin (1980) observed up to 70 % increase in the viable bacterial count in rumen contents treated with 0.1 % Tween 80.

One of the important parameters governing the rate of enzymatic hydrolysis of cellulose is the enzyme (or bacterial) adsorption to its solid substrate. Kudo *et al.* (1987) found that though individual species of cellulolytic organisms differ in both their adsorption mechanisms and their cellulase composition, factors that affect their physical association also affect cellulose digestion. Microbial attachment to cellulose and other fibrous components of the ration is, therefore, of paramount importance. The effects of various levels of Tween 80 on microbial protein adsorption to finely ground straw are shown in Figure 3.2 and is summarized in Table 3.2. Although 0.05 and 0.10 % Tween 80 in the reaction mixture increased microbial protein adsorption ($P < .05$), the effect was not increased further at 0.25 or 0.50 %. Mixed microbial enzyme proteins consist of proteases, cellulases, other enzymes and unlysed bacterial cells. Hence, a mixture of these proteins would have adsorbed to the barley straw. However, the adsorption of cellulases usually parallels the rate of hydrolysis of cellulose (Lee, *et al.*, 1982; Beltrame *et al.*, 1982). Hence, increased cellulase attachment at concentrations up to 0.25 % Tween 80 may have contributed to the significantly higher rate of cellulase degradation shown in Table 3.3. These results differ from findings by Akin (1980) who evaluated the release of rumen bacteria from Bermuda grass cell walls treated with 0.1

% Tween 80. He found that Tween 80 released bacteria from fibre, but observations from electron microscopy indicated that many rumen bacteria remained adhered to the forage cells even after the treatment. His shaker bath operated at 2.5 rps, a rate that would be much higher than the corresponding rumen contractions if Tween 80 was fed to ruminants. Further, gentle physical methods such as shaking have been employed to extract rumen bacterial enzymes (Kopecny and Wallace, 1982).

As previously indicated, the ultimate test of Tween 80's impact would be an in vivo digestibility trial. Table 3.4 shows the chemical composition of the hay fed to sheep in a trial designed to evaluate the effect of Tween 80 on intake and digestibility. A medium quality grass hay was used so that a shortage of protein would not limit rumen function. As the results of this trial showed, (Table 3.5), Tween 80 enhanced feed intake and digestibility ($P < .05$) above the control values. There was almost a 40 % increase in DM intake and approximately 10 percentage units increase in apparent nutrient digestibility, respectively. It should be noted though, that Tween 80 was included at about 0.5 % in the hay ration. This was the calculated concentration from in vitro tests that would elicit maximum microbial activity (Table 3.1). The consumption of water would have a diluting effect on the Tween 80 concentration in the digesta. Since it was found (Table 3.1) that the maximum effective concentration of Tween 80 in vitro was 0.5%, it would be expected that the in vivo response obtained would be lower than the expected potential due to dilution effect. The effect of Tween 80 on anaerobic fermentation has been tested in reactors with subsequent increases in saccharification as a result of increased cellulase activity (Castanon and Wilke, 1981;

Helle *et al.*, 1993) and in biogas production with increases in methane production (Madamwar *et al.*, 1991).

The observed improvement in digestive efficiency noted above was also coupled to increased feed intake. Normally, increased feed intake is associated with a more rapid digesta flow rate and a subsequent reduction in cell wall digestibility (Van Soest, 1990). Since both intake and digestibility increased together it is apparent that Tween 80 improved the efficiency of the digestive enzymes, particularly in the rumen. To confirm whether indeed Tween 80 had an effect on the rumen kinetics, passage rates should be determined using dual-phase markers. The potential of Tween 80 has been further established in that increased milk production has also been observed when 1 % Tween 80 was incorporated into the concentrate portion of the ration fed to lactating dairy cows (J.A. Shelford, 1993 - unpublished data).

3.5 CONCLUSIONS

From the evidence provided above, Tween 80 at concentrations less than 0.5 % enhanced in vitro rumen fermentation. In addition, 0.5 % Tween 80 added to hay also increased its digestibility. However, since most of the effects were localised in the rumen, further investigations on the impact of these surfactants on digestion kinetics is required. Since an increased in vitro protease activity was observed, critical consideration of the ruminal protein digestion efficiency is warranted. The control of ruminal protein degradation could be achieved by masking microbial CYS-SH groups. Hence, the positive attributes of Tween 80 may be enhanced by the inclusion of anionic micelles, e.g. SDS or Teepol HB7.

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Chapter 4

MODIFICATION OF PROTEIN DEGRADATION IN THE RUMEN

4.0 ABSTRACT

The objective of this study was to determine the effects of Teepol HB7 on mixed rumen protease activity, reactive thiols (-SH) and disulfides (-SS), cellulase activity, rumen protein degradation, feed intake and forage digestibility with cows. Results from in vitro protease activity and reactive thiol (-SH) and disulfide (-SS) assays showed that up to 0.3 % (vol/vol) Teepol increased -SH masking ($P < .05$). This was also coupled to a significant ($P < .05$) increase in SS content. The rates of cellulose degradation for the control and Teepol treatments (at 0.10 and 0.3 %) showed no differences ($P < .05$). Apparent microbial enzyme protein adsorption revealed a similar trend to that of cellulose degradation. Tests with 0.5 mM PCMB (p-chloromercuribenzoate - an -SH inhibitor) in the final reaction mixture, resulted in about 80 % inhibition of the proteolytic activity. Although, apparent microbial enzyme protein adsorption was reduced ($P < .05$) by PCMB, cellulose degradation was not affected ($P > .05$). In sacco tests did not show significant differences in effective degradation of alfalfa hay and soybean meal protein with 0.1 % Teepol in the drinking water of the test cows. However, the effective degradation of canola meal protein was reduced ($P < .05$) from 48.7 to 46.4 %. Degradation of rumen fluid soluble protein was reduced by more than 40% with 0.1 % Teepol in the reaction mixture. The rate of

change in the rumen ammonia nitrogen concentration was reduced ($P < .05$) from 1.31 to 0.37 mg/100 ml/h with 0.1 % Teepol in the drinking water of cows fed alfalfa hay. DMI for the control and Teepol treatment were 2.04 and 2.06 (as % body weight), while the apparent digestibilities of DM, ADF, NDF, and CP were 62.38, 47.53, 59.40, 68.08, and 63.48, 49.43, 60.21, and 70.93 (%), respectively. These parameters were not significantly ($P < .05$) different. The decreases in protease activity and protein degradation were attributed to masking of the catalytically active -SH residues. It was also evident from the results obtained that cellulase activity was not inhibited by PCMB and 0.1 % Teepol. The absence of -SH groups in critical steps of the cellulase system was therefore predicted. It was also concluded that low levels of Teepol (< 0.2 %) would decrease the rate and extent of protein degradation in the rumen.

(**Keywords**: protein, rumen, surfactant, degradation)

4.1 INTRODUCTION

With the onset of lactation, a rapid increase in protein requirement arises in ruminants which cannot be met by rumen microbial protein alone. To cater for this need, high quality protein supplements are often fed to lactating dairy cows. However, extensive ruminal proteolysis normally follows, converting the dietary protein to ammonia. Some of the ammonia diffuses from the rumen and is excreted from the body as urea. That the quantity of protein and amino acids delivered to the small intestines normally limits productivity of these animals was shown by their positive responses on postruminal supplementation (Clark, 1975; Broderick *et al.*, 1991). Evidently, it is important to control both the rate and extent of degradation of the dietary protein in the rumen.

The control of dietary protein breakdown in the rumen has received considerable research effort. However. the inhibition of microbial proteases is one area that has received little attention. In fact as late as 1991, some researchers dismissed this strategy as not holding much promise in regulating proteolysis due to the little biochemical data available (Broderick *et al.*, 1991). Yet biochemists were constantly manipulating similar functional and enzyme groups particularly in food processing. In studies of rumen proteases four major classes of proteases (serine-, cysteine-, aspartic-, and metallo-proteases) were identified by Wallace and Cotta, (1988). These authors concluded that the predominant type of protease (56-89 % of the activity) present in the rumen contents, mixed rumen bacteria, and extracted capsular enzymes was a cysteine-protease (CYS-SH) type, which is sensitive to p-chloromercuribenzoate

(PCMB). The other types of activity present were more variable in their contribution to proteolytic activity. These included phenylmethanesulphonyl fluoride (PMSF)- sensitive serine protease (present at 0-41 % of total activity), metalloprotease (9-30 %) and aspartic protease (2-15 %) (Brock *et al.*, 1982; Kopecny and Wallace, 1982; Prins *et al.*, 1983; Wallace, 1984; Wallace and Brammall, 1985). Hence, the strategy of inhibiting rumen proteases to control ruminal protein degradation would entail understanding and subsequently manipulating the -SH groups of the digesta enzymes.

The chemistry of cysteine proteases was discussed at length by Rich (1986). The -SH groups in most cysteine proteases participate in chemical reactions either as thiolate (mercaptide, RS^-) ions (Rich, 1986) or free thiols (Klein and Kirsch, 1969 a,b). A variety of factors could influence the reactivity of these groups within protein macromolecules. An important factor affecting the ionization (reactivity) of the -SH groups in protein molecules is the electrostatic influence of its charged neighbours. Heitmann (1968) found that the incorporation of -SH groups into anionic micelles leads to their "masking", i.e. a sharp decrease in their reactivity. It would be expected that at nondenaturing concentrations anionic surfactants would lead to masking of the catalytic -SH groups of ruminal proteases.

This work was designed to study the effect of Teepol HB7 (an anionic surfactant) on mixed rumen protease and cellulase activity, and rumen protein degradation and digestibility.

4.2 MATERIALS AND METHODS

4.21 Experiment 1. Determination of protease inhibition, thiol and disulfide reactivity and apparent microbial enzyme protein adsorption

4.211 Animals, feed and rumen fluid collection

Four rumen-fistulated, nonlactating Holstein cows averaging 684 ± 48.0 kg in weight were offered 5 kg of medium quality alfalfa hay plus 2 kg of concentrates twice daily. Rumen fluid (1.5 L) was collected from each cow through the fistula 4 h after the morning feeding and homogenized using a Polytron (Brinkmann Instruments, Rexdale, Ontario). The coarse feed particles were removed by sieving the fluid through a 0.5 mm strainer followed by centrifugation at 500 gmax (for 10 min). The microbial pellet was obtained by centrifuging the supernatant obtained at 28,000 gmax for use in the preparation of a microbial enzyme protein powder. The supernatant was saved for the protein degradation estimates (Experiment 3, below)

4.212 Preparation of microbial enzyme protein powder

The microbial enzyme protein powder was prepared using the acetone-butanol extraction procedure outlined by Mahadevan *et al.* (1987). About 300 - 500 g of this powder was prepared and stored at - 20 °C. Extraction of the proteases was accomplished by stirring 250 g of the powder with 1 L of 4 °C cold water for 1 h and then by following Mahadevan's extraction procedure. Extracts from the filtration with an XM-300 Amicon Filter, (approx. 300,000 molecular weight cut off - under nitrogen gas), were made, washed twice with distilled water and the retentate freeze dried (lyophilized). This was referred to as the mixed microbial enzyme protein as it consists

of mixed ruminal enzymes. It was used in the protease activity, protein adsorption tests and also in the thiol reactivity determinations.

4.213 Determination of protease inhibition, thiol and disulfides reactivity

The protease activity -SH and -SS reactivity were assessed in the presence of 10 levels of either Teepol HB7 (pfs grade, Sigma Chemical Co., St. Louis, MO, USA) or PCMB. Mixed microbial enzyme protein (20 g) was dissolved in 200 ml of warm (37 °C) 0.1 M phosphate buffer pH 6.8 and used as a microbial enzyme protein inoculant. The final reaction mixture contained 1 ml of the microbial enzyme protein solution, 1 ml of 2 % casein solution in 0.1 M phosphate buffer and 2 ml of the relevant surfactant in 0.1 M phosphate buffer or its blank (an equivalent amount of the same buffer). Stock solutions of Teepol HB7 (%) and PCMB (mM) were made, (with 0.1 M phosphate buffer), to provide the following test concentrations in the final reaction mixture, viz. 0, 0.025, 0.05, 0.075, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.75.

All incubations were performed in triplicate in 50 ml plastic centrifuge tubes, at 37 °C under a stream of carbon dioxide gas. After 1.5 h, 2 ml of the assay mixture were pipetted out for the determination of reactive thiol (CYS-SH) content, reactive disulfides (-SS, Sasago *et al.*, 1963) and true protein (Smith et al., 1985). At the end of 2 h incubation, the reaction was stopped with 1 ml of 15 % (wt/vol) TCA (trichloroacetic acid), cooled to 4 °C in an icebath, and centrifuged at 10,000 gmax for 10 min. The amino acids and ammonia in the supernatant were assayed using the ninhydrin method (Rosen, 1957). Proteolytic activation (%) was calculated as the increase in amino acids (AA) released (glycine equivalent) per h expressed as a percent

of the AA released by the control incubation over the same experimental duration. Hence, proteolytic activation of the control treatment was 0%. The concentration of the surfactant that gave 20% protease inhibition was considered optimal for inclusion in in vitro cellulose and in vivo protein degradation tests.

Reagents for CYS-SH and -SS were prepared as indicated by Sasago et al. (1963). However, the corresponding level of Teepol HB7 was again added to reestablish the surfactant concentration in the final reaction mixture. Hence, the following solutions were used.

No.	*digesta (ml)*	*PCMB (ml)*	*Teepol (ml)*	*Water (ml)*	
1	-	-	0.5	0.50	(Blank for No. 4)
2	0.25	-	0.5	0.25	(Blank for No. 3)
3	0.25	0.25	0.5	-	
4	-	0.25	0.5	0.25	

CYS-SH was calculated from the value of optical density (OD) at 620 mμ.

OD = (OD1 - OD4) - (OD2 - OD3) using a UV spectrophotometer.

For the estimation of -SS, 0.05 ml of the in vitro digesta, 0.1 ml of 1 M Na_2SO_3, and 0.1 ml of Teepol HB7 (with the twice the test concentration) were further made to 1.45 ml with the corresponding test Teepol HB7 concentration. Na_2SO_3 cleaves CYS-in -SS, thus allowing for their determination following the method outlined for -SH. The determination of -SS then proceeded as for the CYS-SH (Sasago *et al.*, 1963). The standard curve for the PCMB-D reaction was prepared by adding graded amounts of PCMB to dithizone and measuring decrease in optical density (Sasago *et al.*, 1963). The decrease in estimated reactive CYS-SH and -SS content due to increase in the surfactant concentration was referred to as masking CYS-SH and -SS reactivity.

4.2131 Determination of apparent microbial enzyme protein adsorption

The effect of Teepol HB7 and PCMB on apparent microbial protein adsorption was studied by suspending a cellulose substrate (barley straw, with 4 % CP, ground through 0.5 mm sieve) in a solution of microbial enzyme protein. A mother suspension containing 4.0 g of lyophilized mixed microbial enzyme protein in 400 ml of 0.1 M phosphate buffer pH 6.8, with each of the following levels of Teepol HB7; 0, 0.05, 0.1, and 0.3% and 0.5 mM PCMB; was made. Apparent microbial protein adsorption was estimated by stirring, (at 120 strokes/min at 37 °C), 0.1 g of barley straw in 5 ml of a microbial enzyme protein solution, and then following the supernatant protein concentration change with time. The following stirring periods were used; 0, 10, 20, 30, 60, and 120 min. All the determinations were carried out in triplicate and replicated three times. At the end of the stirring period, 1 ml of the assay mixture was pipetted into a 5-ml test tube and centrifuged at 2500 gmax for 10 min to precipitate the solids. The supernatant was decanted, and the protein precipitated by 15 % (wt/vol) TCA. The precipitate was then freeze dried, and protein quantified by the bicinchoninic acid method (Smith *et al.*, 1985). The rates of apparent protein adsorption were calculated by regressing the adsorbed microbial enzyme protein against time. The extent of microbial enzyme protein adsorption was calculated at 120 min stirring period.

4.22 Experiment 2. Determination of cellulose degradation

A bacterial fraction, for testing the effect of Teepol HB7 and PCMB on cellulose degradation, was prepared according to the Forsberg (1978) procedure. This involved centrifuging rumen fluid (digesta) as in experiment 1 above. The digesta pellet was washed, (with 0.1 M phosphate buffer, pH 6.8), and resuspended with an equivalent amount of 0.1 M phosphate buffer pH 6.8 and the rumen fluid supernatant discarded. The resuspended digesta provided both the substrate and the enzyme used for the cellulose degradation assay. The reaction mixture contained 2 ml of resuspended rumen digesta, the test additive or 2 ml of phosphate buffer (blank). The concentration of both Teepol HB7 and PCMB in 0.1 M phosphate buffer were adjusted to accommodate dilution, thus providing testing at 0, 0.05, 0.1 and 0.3% Teepol HB7 (vol/vol) and 0.5 mM PCMB in the final reaction mixture.

Incubations were carried out at 37 °C for periods of 0, 1.5, 3, 6, 12, 24, and 48 h in 50 ml centrifuge tubes under a stream of carbon dioxide. All incubations were carried out in triplicate and replicated three times. The initial and final concentrations of cellulose were determined by the method of Updergraff (1969). Microgranular cellulose was used at graded levels in the preparation of a standard curve. The initial rates of cellulose degradation were calculated by regression analyses.

4.23 **Experiment 3. Determination of protein degradation (in vitro and in sacco) and in vivo digestibility trial**

About 4 L of the supernatant fluid obtained from Experiment 1 above were filtered through the YM-10 Amicon filter at 4 °C, (approx. 10,000 mol. wt. cut-off). The filtrate, referred to as digesta fluid, was autoclaved at 120 °C for 10 min, cooled, and used to extract alfalfa, canola and soybean meal protein.

Ten tightly closed Dacron bags (12.5 x 5 cm, with 40 µm pore size), containing about 1.5 g of each feed sample were immersed in 15 ml of the prewarmed 37 °C digesta fluid in 50 ml centrifuge tubes and agitated at 120 strokes/min at 37 °C for 1 h. At the end of the agitation (extraction) period, all the contents including the bag were carefully filtered under vacuum onto a buchner funnel with a glass receiver. The filtrate was further filtered through the YM-10 Amicon filter. After washing once with distilled water, the retentate was freeze dried. This fraction is equivalent to the 'a' fraction in the nylon bag procedure (Orskov *et al.*, 1980) and its protein content was referred to as the rumen fluid soluble protein.

The in vitro incubation procedure of Mahadevan *et al.* (1987) was used to determine the degradation rates of the freeze dried rumen fluid soluble proteins. Rumen fluid soluble proteins (0.01g), were incubated with 2 ml of mixed microbial enzyme protein solution and 2 ml of 0.2 % (vol/vol) Teepol HB7 (in 0.1 M phosphate buffer, pH 6.8). In the control treatment, 2 ml of buffer replaced the surfactant in the final incubation mixture. The released ninhydrin positive material (amino acids and ammonia) was estimated as mg glycine equivalent using the procedure outlined by

Rosen (1957). Except for the incubation periods (which were 0, 2, 4, and 6 h), the incubation conditions and procedures outlined in Experiment 1 were used. The initial rates of protein degradation (Vda) were calculated by regressing AA released (glycine equivalent) against the time of incubation.

4.221 Effect of Teepol HB7 on protein degradation and in vivo digestibility trial

The effects of Teepol HB7 on feed intake and digestibility were estimated using four cows weighing 684 ± 48.0 kg liveweight fitted with rumen fistulae and duodenal-T cannulae. The animals were housed in individual stalls (sawdust covered rubber floors) during total collection, and in group pens during the adaptation period. Ten kg of alfalfa hay were offered in two equal portions, at 08:30 and 20:30 h. Water was available ad libitum from 150 L plastic bins. The Teepol treatment consisted of 1 ml Teepol HB7 / 1 L of drinking water. The inclusion level of the surfactant was based on the results from in vitro protease inhibition and cellulose degradation studies mentioned above viz., the level that resulted in about 20% protease inhibition without adverse effects on cellulose degradation. The experiment was designed as a 2x2 latin square with two sources of water (treated or untreated) and two groups of animals. The experimental periods consisted of a 14-day adaptation and a 7-day collection.

Sample collection and the relevant analyses followed the same procedures and methods as in Experiment 3 of Chapter 3 above. The Teepol-treated water was changed every second day when the level was below 20 L.

The effect of Teepol HB7 on protein degradability (Nylon bag technique - Orskov *et al.*, 1980) was estimated from d-10 of the adaptation period on the following

feeds; alfalfa hay, canola and soybean meal. Up to 5 g of the test material were weighed into Dacron bags, (12.5 x 5 cm, 40 μm pore size) and incubated for 0, 6, 12, 18, 24, 36, 48, and 72 h in each of the four cows. At the end of the incubation period, the bags were retrieved and rinsed (4 cycles with cold water, Maytag washing machine) and dried (60 - 80 °C to constant weight). DM and CP content were then determined as in Experiment 3 of Chapter 3. Other analyses of the data obtained followed the procedure used for evaluation of CP degradation in concentrates by Orskov et al. (1980) and Kamande (1988). The degradation constants as outlined by Orskov *et al.* (1980) were derived, viz., a- soluble fraction (%), b- potentially degradable fraction (%), c- the rate of degradation of the fraction 'b' (%/h); Pf_b (%)- effective protein degradability corrected for the rate of passage of the 'b' fraction; $RUDP_b$ (%)- undegradable protein fraction given by ($100-Pf_b$).

Sampling for in vivo rumen ammonia concentration was done at 0, 1, 2, 3, 4, 6, and 12 h post-feeding on d 10 of the adaptation period. Approximately 100 ml of rumen fluid were collected from each cow and a 5 ml portion mixed with 1 ml of 25 % metaphosphoric acid (wt/vol) containing 0.025 M isocaproic acid (internal standard) and kept frozen in tightly capped tubes. Ammonia in the supernatant (50 μL) was determined colorimetrically by the hypochlorite method (Wetherburn, 1967). The rates of change of ammonia nitrogen concentration were estimated by regressing concentration (mg/100 ml) against time (h).

4.24 Statistical analyses

Data were analyzed as a factorial experiment using the PROC GLM of the SAS Statistical Package (1985), and the means were compared by the Tukey's test. The level of significance was set at ($P < .05$). Similar statistical models and procedures for analyzing the degradation rates (regression analyses) as indicated in Chapter 3 were employed.

4.3 RESULTS

4.31 Experiment 1.

4.311 Protease inhibition and reactive -SH and -SS

Proteolytic inhibition of reactive thiol and disulfide content with varying levels of Teepol and in the presence of PCMB is shown in Figures 4.1 and 4.2, respectively. Significant ($P < .05$) increases in proteolytic inhibition were observed from 0.05 to 0.2 % Teepol in the final incubation mixture. Over the same levels of the surfactant, masking of the reactive CYS-SH content and disulfide reactivity increased ($P < .05$) (Fig 4.2). Proteolytic inhibition increased (Fig 4.1) with increasing masking of -SH reactivity (Fig 4.2) between 0.3 to 0.5 % Teepol. With excess PCMB (>0.45 mM, Fig 4.1), up to 80% of the protease activity was inhibited by PCMB. Inhibition of protease activity by PCMB (Fig 4.1) corresponded with increased interaction (binding) between reactive -SH and PCMB (Fig 4.2).

4.312 Apparent microbial enzyme adsorption.

The profile of the apparent microbial enzyme protein adsorption is shown in Figures 4.5 and 4.6. Rates of apparent microbial enzyme protein adsorption showed a significant ($P < .05$) increase at 0.05 % Teepol compared to the other Teepol treatments (Table 4.1). The rate of adsorption declined at levels above 0.05 % Teepol (Table 4.1). This effect was reversed at 0.1 and 0.3% Teepol, which was not different ($P < .05$) from the control. However, PCMB reduced the rate of apparent microbial enzyme protein adsorption significantly ($P < .05$).

Table 4.1. Effect of Teepol HB7 or PCMB on the rates of apparent microbial enzyme protein adsorption

Treatment	adsorption rate	s.d.
no additive	9.4ab	(0.6)
0.05 % Teepol	10.1a	(0.3)
0.10 % Teepol	8.2bc	(0.8)
0.30 % Teepol	8.3bc	(0.2)
0.5 mM PCMB	6.9d	(0.3)

Values followed by different letters are diffrent ($P < .05$). s.d. = Bracketed values are standard deviations. n=3. Adsorption rate (10^{-3}x μg/mg/min)

4.32 Experiment 2. Effect of Teepol HB7 on cellulose degradation

PCMB did not affect cellulose degradation significantly ($P < .05$). Figures 4.3 and 4.4 show the in vitro cellulose degradation profile with Teepol and PCMB respectively. The initial cellulose degradation rates were computed by regression analysis from the values on the initial 6 h incubation and are shown in Table 4.2. The in vitro degradation of cellulose showed a significant rate increase with 0.05 % Teepol. However, at 0.1 and 0.3 % Teepol, the initial rate of cellulose degradation was similar to that of the control.

Table 4.2. Effect of Teepol HB7 and PCMB on the rates of cellulose degradation

Treatment	degradation rate	s.d.
no additive	2.39a	(0.23)
0.05 % Teepol	3.04b	(0.31)
0.10 % Teepol	2.31a	(0.30)
0.30 % Teepol	2.10a	(0.23)
0.5 mM PCMB	2.27a	(0.10)

Values followed by the different letters are different ($P < .05$). Bracketed values are standard deviations. n=3. Cellulose degradation rate (μg/ml/h).

4.33 Experiment 3. Effect of Teepol HB7 on protein degradation, feed intake and digestibility

At 0.1 % Teepol in the in vitro assay mixture, about 20 % inhibition in protease activity was observed. Since, no detrimental effect on cellulose degradation was noted, this level was adopted in the subsequent in vitro protein degradation assay, (using rumen digesta fluid-soluble alfalfa hay, canola meal protein, and soybean meal protein), and in both the in sacco and the in vivo digestibility trials.

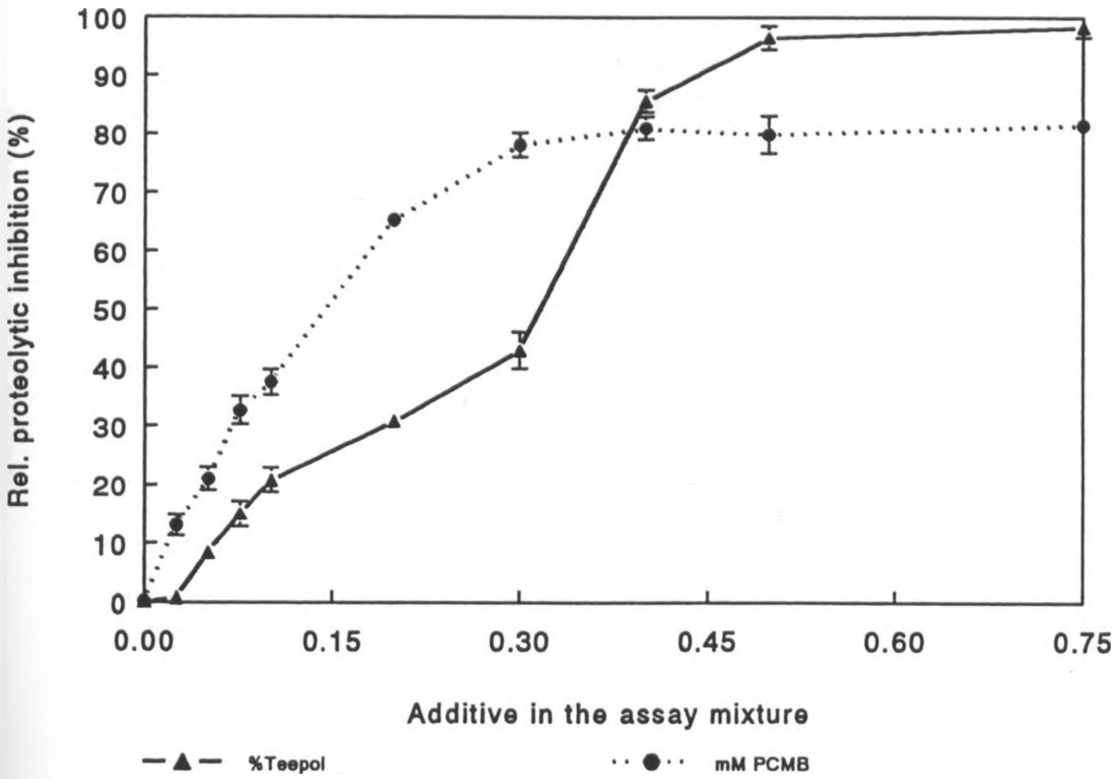

Fig 4.1 Effect of Teepol HB7 and PCMB on rumen protease inhibition

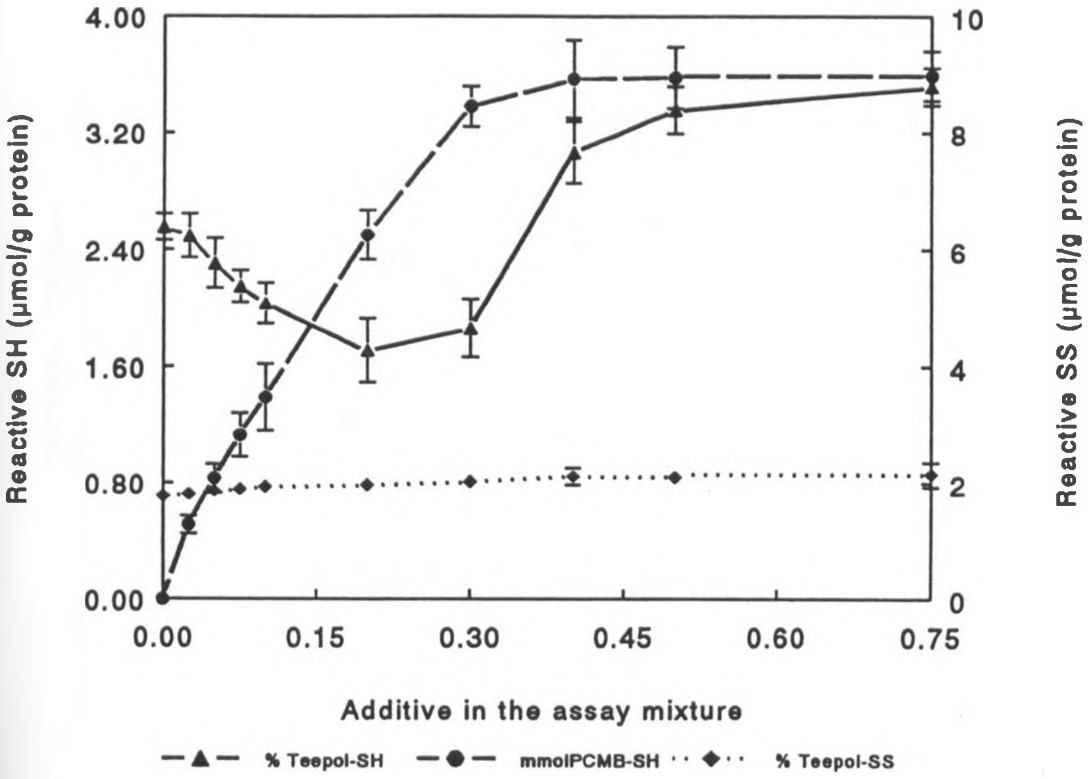

Fig 4.2 Effect of Teepol HB7 and PCMB on the reactivity of ruminal -SH and -SS

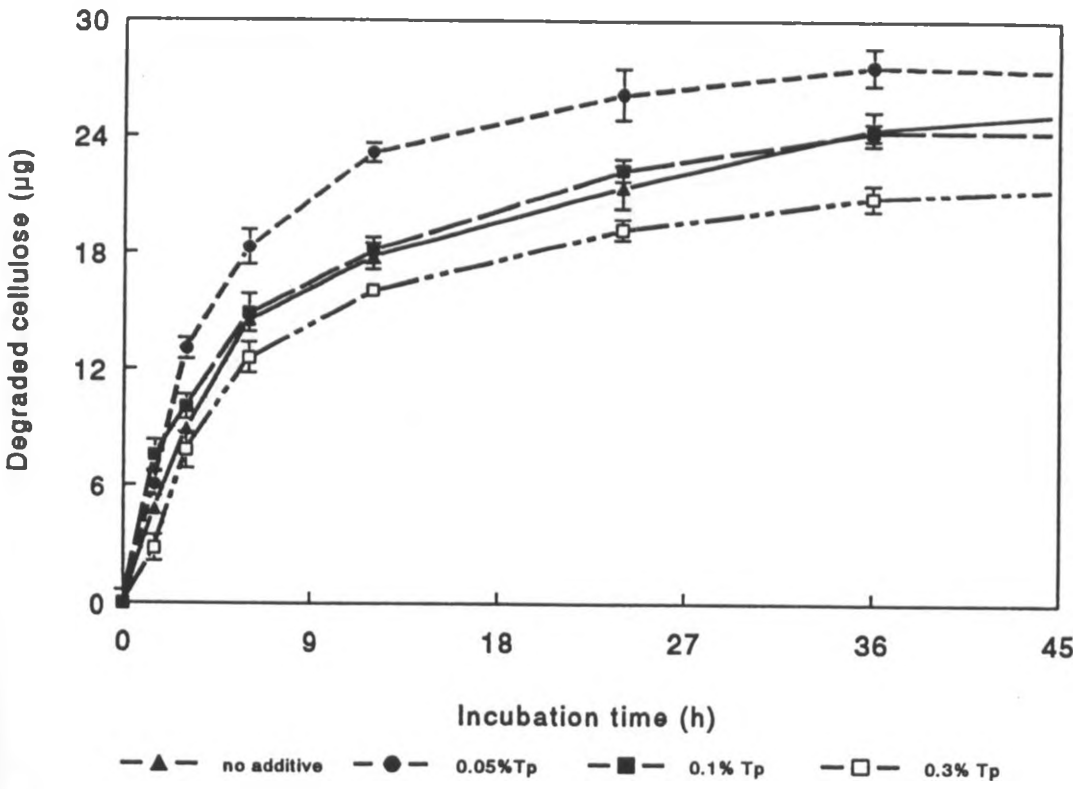

Fig 4.3 Effect of Teepol HB7 on in vitro degradation of cellulose

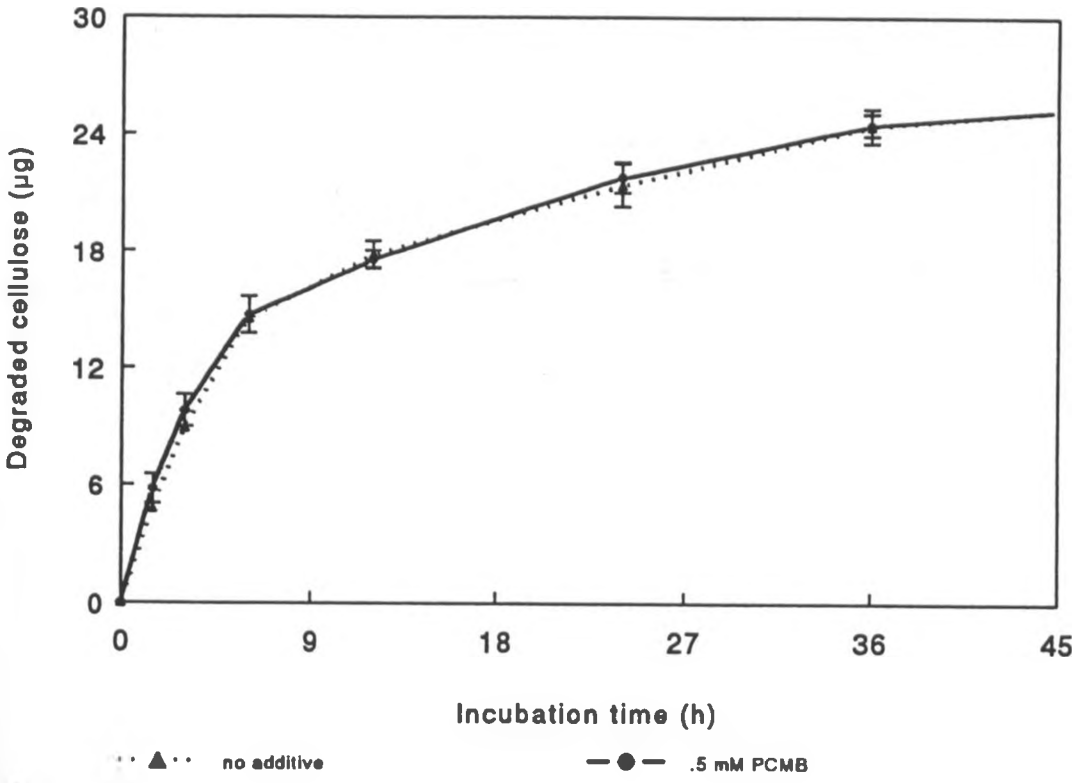

Fig 4.4 Effect of PCMB on in vitro degradation of cellulose

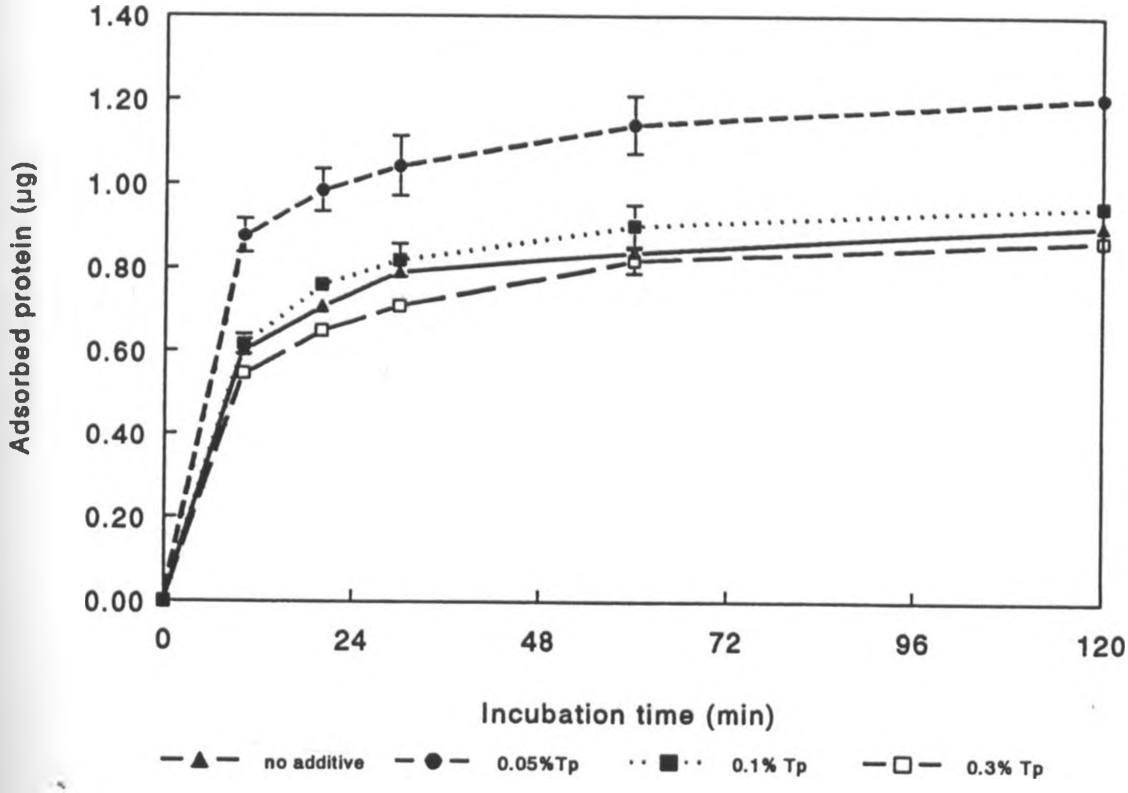

Fig. 4.5. Effects of Teepol HB7 on apparent microbial enzyme protein adsorption

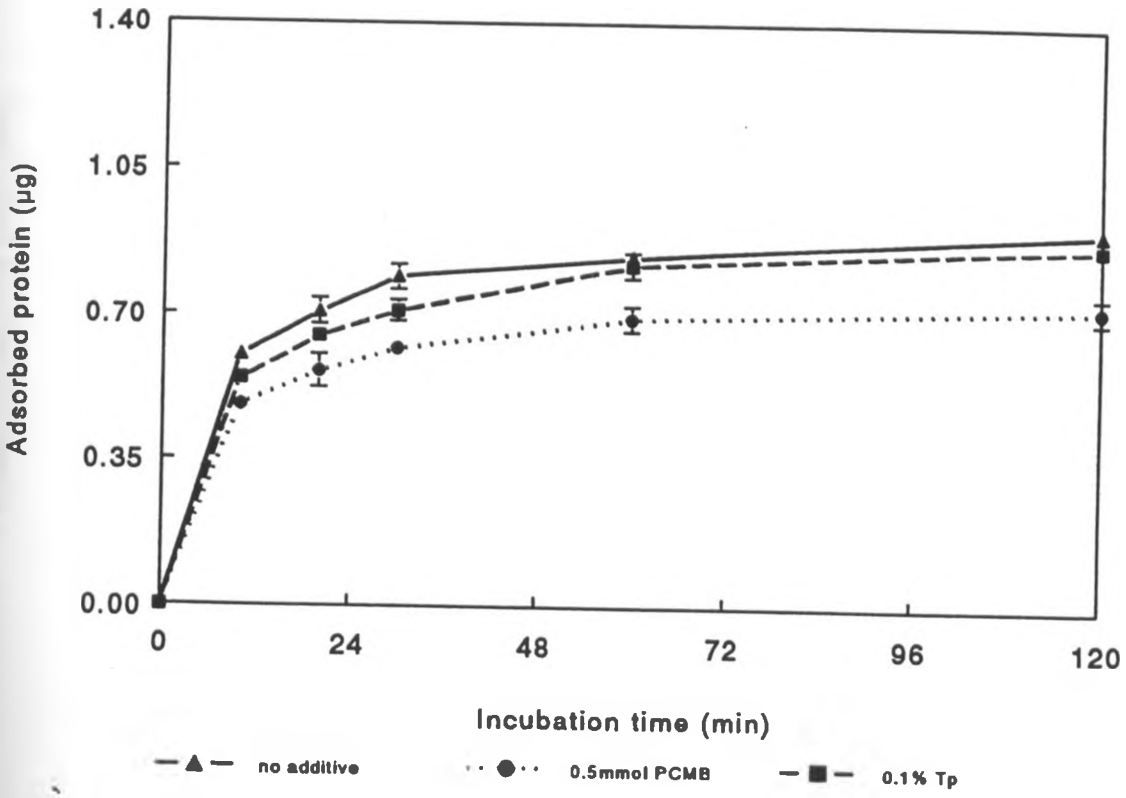

Fig. 4.6. Effect of Teepol and PCMB on the adsorption of microbial enzyme protein

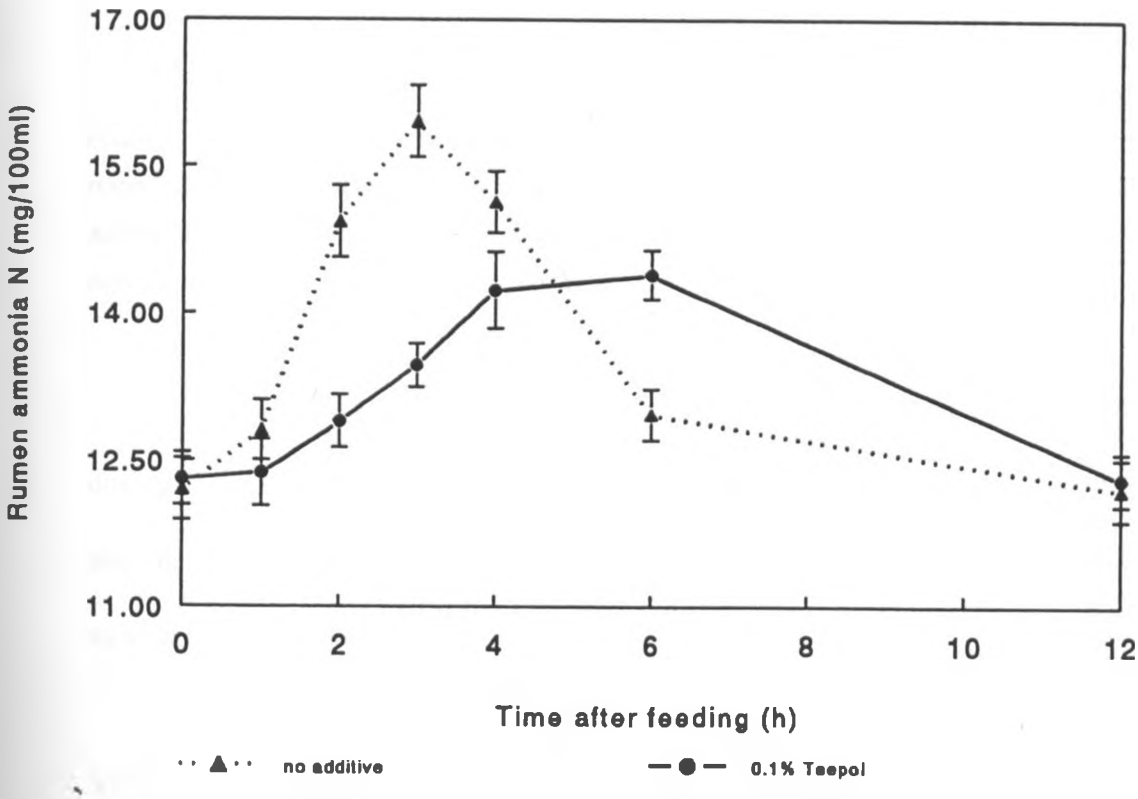

Fig. 4.7. Effects of 0.1% Teepol in the drinking water on rumen ammonia N

Table 4.3 shows both the initial rates of degradation of rumen fluid soluble proteins (v_{da}) and the nylon bag degradation constants.

Table 4.3 In vitro and in sacco protein degradation constants of canola, soybean and alfalfa supplements

Feed Treatment	v_{da}		a	b	c	Pf_b		$RUDP_b$	
CAN control	3.5a	(.01)	27.8	44.9	4.3	48.7a	(.30)	51.3a	(.30)
CAN Teepol	2.5b	(.02)	30.7	41.9	2.9	46.4b	(.74)	53.6b	(.74)
SBM control	5.3a	(.01)	30.3	62.0	4.6	60.2a	(.40)	39.8a	(.40)
SBM Teepol	3.2b	(.04)	35.5	56.7	3.6	59.5a	(1.2)	40.5a	(1.2)
ALF control	2.7a	(.02)	19.7	61.7	3.9	47.1a	(.67)	52.9a	(.67)
ALF Teepol	1.8b	(.03)	25.1	56.7	3.1	47.1a	(.47)	52.9a	(.47)

Feed group values within columns followed by different letters differ ($P < .05$). Standard errors of the respective means obtained from n=3 in each of 4 replicates (cows) are enclosed in brackets. **Abbreviations**: CAN- canola meal; SBM- soybean meal; ALF- alfalfa hay; v_{da}- in vitro protein degradation rate of the rumen fluid extractable fraction (amino acids released mg/2h).

The ammonia nitrogen concentration in the rumen fluid samples collected during the in vivo trial are shown in Figure 4.7. The rates of ammonia-N accumulation shown in Table 4.4 were calculated by regressing rumen ammonia concentration against sampling time.

Table 4.4 Initial rates of change in the rumen ammonia N concentration

Treatment	rate (mg/100 ml/h)	
no additive	1.57a	(0.32)
0.1 % Teepol	0.92b	(0.19)

Values followed by different letters are different ($P < .05$). Bracketed values are standard deviations obtained from n=12 in each of the 4 (cows) replicates.

Other in vivo parameters investigated included feed intake and apparent nutrient digestibility. Table 4.5 shows the feed intake and digestibility coefficients obtained while feeding alfalfa hay and providing water treated with 0.1 % Teepol. There was no significant difference ($P < .05$) in DM intake and nutrient digestibility between the control and the Teepol diet. Since animals on the Teepol treatment consumed about the same amount of water per day (40 L) as those in the control group, the additive was considered adequately delivered.

Table 4.5 Effects of 0.1% Teepol HB7 in drinking water on feed intake and digestibility

	no additive	0.1% Teepol	S.E.M
DM Intake[1]	2.04a	2.06a	(0.11)
Apparent digestibility (%);			
Dry matter	62.38a	63.48a	(0.62)
Acid detergent fibre	47.53a	49.43a	(0.74)
Neutral detergent fibre	59.40a	60.21a	(0.66)
Crude protein	68.08a	70.93a	(0.75)

Values within rows followed by different letters differ ($P < .05$). Bracketed values are standard errors of the respective means (for each of the 4 replicates (cows)). [1]DMI - as % of BW.

4.4 DISCUSSION

Inhibition of cysteine proteases by greater than 0.3 mM of PCMB accounted for a loss of almost 80 % of the total rumen protease activity as shown in Figure 4.1. This concurs with the view that this is the predominant protease group in the rumen (Wallace and Cotta, 1988),. Further, this suggests that -SH groups are involved either in the catalytic site, the enzymic substrate binding site or in vital step(s) of enzyme catalysis. None-the-less, their significant involvement in proteolysis provides an opportunity for their modification and consequently in the manipulation of rumen protein degradation.

Rich (1986) discussed the mechanism of action of cysteine proteases. When the -SH groups of cysteine proteases participate in catalysis in the ionized form (RS^-), the electrostatic influence of its charged neighbours has a tremendous influence on their reactivity (ionization). Heitmann (1968) found that the incorporation of -SH groups into anionic micelles leads to a sharp decrease in their reactivity. The significant increase in -SH masking between 0.05 to 0.2 % Teepol, an anionic surfactant, is in agreement with findings by Sasago *et al.* (1963) and Heitmann (1968). The former observed a decrease in the reactive -SH groups of milk with 0.25 % sodium dodecylsulfate (SDS). The slight increase in the disulfide content observed with increased -SH masking was associated with a tendency for -SH oxidation, thus increasing structural stability within the enzyme protein molecule while decreasing both the -SH reactivity and consequently the protease activity.

The increased -SH content observed at >0.3 % Teepol in the assay mixture would be the result of structural unfolding (denaturation). The fact that PCMB also significantly reduced microbial protein adsorption indicates that both adsorption site and catalytic site for rumen cysteine-proteases are closely associated or identical. A similar conclusion was arrived at by Wallace (1985).

That a significant reduction in microbial protein adsorption with PCMB treatment did not coincided with reduced cellulose degradation rates (Table 4.2) was not as expected. Microorganisms must attach themselves to their insoluble substrates in order to effect digestion (Cheng *et al.*, 1991). However, it is possible that only the adsorption of cysteine proteases was affected by PCMB and not adsorption of cellulases. Further, this indicates that -SH groups may not be involved in the catalytic and binding sites of the cellulase system. After titrating with dithiobis(2-nitrobenzoic acid), Clarke and Yaguchi (1985) did not establish the presence of free thiol groups in endo-β-1,4-glucanase of Schizophyllum commune. In contrast, they proposed that carboxyl groups are involved in the active site. Nevertheless, Johnson and Demain (1984) had postulated the possible involvement of -SH groups in the cellulase of Clostridium thermocellum. Whether, the catalytic sites of fungal and bacterial cellulases studied are different seems unresolved. It is of interest to mention that, Komai and Noguchi (1971) observed the cooperation between -SH and COO^- (carboxyl) groups in their investigations on the mechanisms of -SH enzymes.

The fact that 0.3 % Teepol decreased cellulose degradation compared to the other Teepol treatments may be a function of denaturation. However, the significant

increase in microbial enzyme protein adsorption at 0.05 % Teepol (Figure 4.5 and Table 4.1) corresponded with increased cellulose degradation and protease inhibition (Figure 4.3 and Table 4.3). It is possible that 0.05 % Teepol enhanced cellulase adsorption to the hydrophobic sites of the cellulose while masking the protease -SH groups. According to Van Soest (1990), plant feed particles normally assume a cation binding capacity in the rumen due to such groups as phenolic, amino, hydroxyls, carboxyl, and free aliphatic hydroxyls. These groups have some affinity toward binding the positively charged metal ions. Their interaction with microbes having negatively charged cell walls require divalent cation liganding. However, at less than the critical micelle concentration, e.g. 0.05 % Teepol, protein-surfactant micelles develop as the surfactant attaches on the surface of the proteins including enzymes. Liganding by the surfactant to the feed particles could establish a close interaction between the cellulases and their substrate. This may have contributed to the improved cellulose degradation rate at 0.05 % Teepol. However, at a higher than the critical micelle concentration, e.g. > 0.1 % Teepol, micelle repulsive forces may hinder further adsorption thus, resulting in reduced cellulase activity.

As the potential contribution to rumen protein bypass was about 20 % (20% protease inhibition occurred with 0.1% Teepol - Fig. 4.1), it was deemed that 0.1 % Teepol be adopted in the subsequent in vivo trials. At this level, no adverse effect on cellulose degradation was observed. Results from in sacco protein degradation are shown in Table 4.3. Of the three protein supplements, only canola meal showed a significant ($P < .05$) reduction in effective protein degradation (Pf_b). Treatment effects

on the other two protein supplements may have been masked by the underlying assumptions of the in sacco method. The nylon bag technique as outlined by Orskov *et al.* (1980) makes two erroneous assumptions that minimize its usefulness. The amount of protein that leaves the bag upon washing or following up to 1 h incubation in the rumen and subsequent washing is considered equal to pool A. Pool A, as measured by this technique consists of soluble proteins and protein residing in very small feed particles that are removed when the bag is washed. Pool A is also assumed to be instantaneously degraded. Hence a k_{d_a} value of 100 % is assumed. The in vitro results shown in Table 4.2 as v_{d_a} values concur with the views of Mahadevan *et al.* (1980) that not all soluble proteins are hydrolyzed rapidly or at the same rate. In fact some of the protein supplement feed particles were small enough to leave the nylon bags after 1 h of agitation. That such a diverse pool is assumed to degrade at a rapid or at the same rate may not be correct. Other researchers also believe that the soluble fraction may not be readily degraded in the rumen (Satter, 1986).

It would be advantageous to be able to correct for the degradation rates of pools A and B. In situations where pool A (Orskov et al., 1980) is large and where treatments influence the molecular breakdown of soluble nutrients, assuming a k_{d_a} value of 100 % would totally mask significant effects. An attempt to further correct k_{d_a} was therefore warranted. From Table 4.3, significant ($P < .05$) treatment effects were observed with in vitro degradation of pool A. Proteins in Pool A should be corrected for their respective degradation rates and related to their proportionate outflow rates.

Hence,

Equation 2; $Pf_b = a + (bc)/(c+k)$ .....(Orskov *et al.*, 1980)

becomes $Pf_{ab} = ((ak_{da})/(k_{da}+k)) + ((bc)/(c+k))$,

The Pf_{ab} calculated would have pools A and B related to their proportionate degradation (k_{da} or c) and outflow rate (k). A similar approach was proposed by Broderick (1994) in his two-compartment model.

The participation of -SH groups in catalysis can be grouped into two major roles; (a) nucleophilic catalysts (Rich, 1986) and (b) dithiol-disulfide conversions in the active sites of oxidoreductases (Barron, 1951; Jocelyn, 1972). Glyceraldehyde 3-phosphate dehydrogenase, (GPDH, EC 1.2.1.12) an enzyme of glycolysis, was the first enzyme for which direct evidence was obtained for the involvement of thiols through the formation of a covalent acyl-thioester intermediate during the catalytic cycle (Krimsky and Racker, 1955). Later the formation of acyl enzymes was shown in the action mechanisms of proteases such as papain. GPDH plays an important role in the metabolism of carbohydrates in most living organisms. In the presence of NAD^+ and phosphate, it catalyses the reversible oxidative phosphorylation of 3-phosphoglyceraldehyde to 1,3-diphosphoglyceric acid. Hence, reducing the reactivity of ruminal enzymic -SH groups might also reduce the degradability of some dietary carbohydrates. It is known that most proteolytic rumen bacteria are also amylolytic in nature. In this study starch degradation was not measured.

As indicated by rumen ammonia nitrogen concentrations the rate of protein degradation with an alfalfa hay diet (Table 4.4) was slowed by almost 40 % with Teepol treatment. Ammonia is the end-product of the deamination of amino acids in the rumen. The inhibitive effect of Teepol on this process may be related again to its reduction of the reactivity of the -SH groups of highly catalytic enzymes in the rumen. It is possible that -SH masking reduced the involvement of -SH groups of oxidative enzymes that act as intermediate electron carriers (especially on the microbial cell surface) from substrates to such acceptors as NAD^+. Russell and Jeraci (1984) and Russell and Martin (1984) found that rumen ammonia production declined primarily as a consequence of inhibition of branched-chain amino acid fermentation. However, in cell extracts of rumen bacteria, the $NADH/NAD^+$ ratio was an important effector of branched-chain amino acid fermentation, with NAD^+ being essential as an electron acceptor. Thus when hydrogenase was inhibited, the $NADH/NAD^+$ ratio increased and amino acid deamination declined (Hino and Russell, 1985). In addition, Kopecny and Wallace (1982), indicated that cysteine protease activity was predominant in a mixed rumen bacterial population, and particularly Bacteroides ruminocola was sensitive to sulfhydryl reagents. These bacterial species were also highly deaminative (Wallace and Cotta, 1988).

Results from the in vivo trial (Table 4.5) showed no significant difference in DM feed intake or apparent nutrient digestibility with 0.1% Teepol in drinking water. Rumen ammonia nitrogen concentrations (Table 4.4) and the in vitro degradation

results showed that protein degradation in the rumen was inhibited. However, since the total tract digestion with 0.1% Teepol treatment was similar to the control, extensive protein digestion in the lower gut may have occurred. Consequently, increased dietary amino acids may have been directly available to the small intestines. The digestion kinetics may not have been affected because feed intake was not significantly different.

4.5 CONCLUSIONS

Since the predominant protease activity in the rumen is CYS-SH type, -SH masking by the use of anionic micelles was attempted. At 0.1 % Teepol, the protein adsorption to cellulose and reactive -SH content of mixed bacterial protein were reduced ($P < .05$). In addition, in vitro degradation results with the same level showed a significant reduction in amino acid release from substrate proteins in the presence of mixed rumen proteases. Although the in sacco technique failed to show differences, the in vivo degradation of model protein supplements, namely alfalfa hay, canola meal and soybean meal, rumen ammonia N concentration values confirmed significant treatment effects with 0.1 % Teepol. The in vitro degradation results showed that rumen fluid soluble proteins from the model protein substrates degraded at significantly ($P < .05$) different rates. This was contrary to the assumption that pool A is instantaneously degraded. In vivo intake and digestibility results showed no significant differences between Teepol treatment and the control alfalfa diet. It was evident from the results obtained that 0.1 % Teepol increased the rumen undegradable protein fraction significantly without adverse effects on the total tract digestibility.

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Chapter 5

EFFECT OF pH AND CHEMICAL INHIBITORS ON THE PROTEASE ACTIVITY OF ORCHARD GRASS DURING ENSILAGE

5.0 ABSTRACT

The objective of this study was to characterise the proteases in ensiled orchard grass according to their reactions at various pH values and with various chemical inhibitors. Two inhibitors, namely PCMB (p-chloromercuribenzoate - a thiol (SH) inhibitor) and PMSF (phenylmethanesulfonyl fluoride - a serinyl and SH inhibitor), were tested. The effect of pH change (4.0 to 7.0) on proteolysis was also investigated. Immature orchard grass was harvested and directly ensiled for 0,1,2,5,9,21, and 45 d at 30 °C in air-tight plastic pails (2.0 kg/pail). At the end of the ensiling period, the silage was ground with a coffee grinder under liquid nitrogen and stored at -50 °C pending further analyses. Each treatment was replicated three times. The rates of proteolysis declined with increasing acidity and ensiling period. These rates ranged from 0.70 ± 0.03 in fresh grass to 5 d ensiling and to 0.38 ± 0.07 (μmol. glycine equivalent/g/h) between 9 and 45 d ensiling. Proteolysis in the latter period could be mainly microbial in origin. PCMB and PMSF inhibited proteolysis by up to 90 % in the first 5 d. Complete inhibition occurred after 9 d post-ensiling with both inhibitors. Results from the pH study showed a significant increase in proteolysis with increased pH for all silages tested. Decreased proteolysis at low pH was attributed to the reduced reactivity

of the protease catalytic site. It was therefore concluded that orchard grass proteases are CYS-SH in nature.

Key words: Orchard grass, proteases, thiol, inhibitors, pH

5.1 INTRODUCTION

Orchard grass is one of the major forages grown in the Fraser Valley of British Columbia and in most of the Pacific Northwest region. It would be a potential source of inexpensive protein for dairy cows were it not for the wet growing season which often precludes making hay during the first cut. Ensiling is widely practised but subsequent proteolysis and the resulting increase in non protein nitrogen (NPN) limits its usefulness in meeting the metabolizable protein requirements of lactating dairy cows.

Although fresh forage contains up to 90 % of its total nitrogen in the form of proteins, wilting and the first week post-ensiling could result in 60 % proteolysis (Ohshima *et al.*, 1979). Extensive protein degradation in the first few days of ensiling is primarily the consequence of unabated activities of plant proteases, followed by bacterial deaminases (Ohshima *et al.*, 1979; McKersie, 1981). Hence, successful silage preservation should involve control measures based on the complete understanding of these degradative activities.

Proteases in all living systems have been classified into four major groups, (Ryan, 1973; Barrett, 1986), depending on their active site catalytic mechanisms. These include the serine-, cysteine-, aspartic- and metallo- proteases. These form the sub-sub-classes 21, 22, 23 and 24, respectively, of the peptide bond hydrolases (subclass 3.4) in the enzyme nomenclature scheme (IUB Nomenclature Committee, 1984). Specific chemical inhibitors have been used to characterize the proteases in alfalfa (McKersie, 1981) and in ten other herbages, (Singh, 1962), excluding orchard grass. From these reports phenylmethanesulfonylfluoride (PMSF), which inhibits both serine and cysteine

(CYS-SH) proteases, and p-chloromercuribenzoate (PCMB) - an CYS-SH protease inhibitor, seem most effective (Bollag and Edelstein, 1991). This is in agreement with the view that plant proteases are cysteinyl (CYS-SH) in nature (Singh, 1962; Glazer and Smith, 1971; Csoma and Polgar, 1984;). Cysteine proteases (EC number 3.4.22) are also found in bacteria, in eukaryotic micro-organisms, and in animals (Barrett, 1986).

Other properties of proteases in leaves and plant extracts have been extensively studied (Singh, 1962; Ryan, 1973; Ragster and Chrispeels, 1981; McKersie, 1981 and 1985). In various studies involving plant proteases, both acid and alkaline proteases, endopeptidases, carboxypeptidases and aminopeptidases have been characterized (Ryan, 1973; McKersie, 1981). These workers also found that below pH 4, proteolytic activity was considerably reduced. Some ensiling technologies have exploited direct acidification to inhibit proteolytic enzymes and reduce silage NPN content. Nevertheless, acids are expensive and corrosive. Hence, there is still need for technologies based on potent forage protease inhibitors.

A clear understanding of proteolytic pathways of the forage proteases, particularly prior to the point where silage pH adequately inhibits proteolysis, is also required. The objective of this project was to study orchard grass silage proteases in relation to the effects of pH and thiol inhibitors.

5.2 MATERIALS AND METHODS

5.21 Silage preparation

Immature orchard grass was harvested (New Holland, Forage Harvester) from Agriculture Canada field plots at Agassiz, B.C. About 2.0 kg of the grass (second cut) were directly packed in airtight plastic pails (2.5 kg capacity) and stored at 30 °C room ambient temperature for 0, 1, 2, 5, 9, 21, and 45 d. Each treatment was replicated three times. At the end of each ensiling period, the silage was mixed and subsampled. A final 200 g sample was obtained and ground under liquid nitrogen (-196 °C) using a coffee grinder. The material was stored at -50 °C pending further analyses. Dry matter (DM, 80 °C to constant weight), crude protein (CP), trichloroacetic acid insoluble CP (Technicon, autoanalyzer), true protein (Smith *et al.*, 1985), lactic acid (Barker and Summerson, 1941) and, water soluble carbohydrates (CHO, glucose was used as a standard) were measured (Dubois *et al.*, 1951). Silage pH was determined by homogenizing 20 g of forage using a Polytron (Brinkmann Instruments, Rexdale, Ontario) in 200 ml of deionized water. The pH reading was taken while the homogenate was being stirred in a beaker using a magnetic bar.

5.21 Forage fractionation

An equivalent of 50 g DM forage was homogenized for 5 min in 100 ml of cold (4 °C) 0.2 M phosphate buffer, pH 6.0 and 10 mM sodium isoascorbate. The homogenate was centrifuged at 10,000 gmax for 10 min at 4 °C. The supernatant was then filtered using a 10,000 molecular weight cut off Amicon YM 10 filter membrane (R. Grace and Co.,

Danvers, MA.). The extract was washed twice with 200 ml of 0.2 M phosphate buffer, freeze dried and stored at -50 °C for later use as the enzyme extract.

5.22 Effect of PCMB or PMSF on orchard grass proteases

The effect of PCMB and PMSF (phenylmethanesulfonyl fluoride) on the protease activity was determined by incubating, under a stream of nitrogen gas, 1 ml of orchard grass extract solution containing 5 g of the extract in 100 ml 0.2 M phosphate buffer pH 6.0 and 10 mM sodium isoascorbate with 1 ml of 4 % casein, 0.5 ml of the inhibitor or 0.5 ml of the buffer at 30 °C for 2h. The exclusion of air by nitrogen was designed to minimize thiol oxidation. Stock solutions (10 mM) of PCMB or PMSF were prepared in isopropanol (Bollag and Edelstein, 1991). A final total volume of 2.5 ml and a concentration of 1 mM inhibitor in the assay mixture was maintained. An equivalent volume of isopropanol was added to the phosphate buffer of the control tubes. At the end of the incubation period, the reaction was stopped by the addition of 2.0 ml of cold 15 % (wt/vol) TCA solution. After cooling for 1 h in an ice bath, the precipitate was removed by centrifugation (10,000 gmax for 10 min). Free amino acids were estimated in 0.1 ml of the supernatant by the ninhydrin method of Rosen (1957). Protease activities were expressed as amino acids (glycine equivalent) released (μmol /g/h) as a percentage of μmol AA released/g/h by the control (0 day ensiling).

5.23 Effect of pH changes on orchard grass protease activity

The effect of pH on proteolytic activity of the extract was determined by mixing 2.5 ml of orchard grass forage extract solution prepared as indicated above with equal volumes of the appropriate pH buffer. Stock solutions of 0.2 M phosphate buffer were

adjusted to pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and, 7.0. Incubations were performed in 50 ml centrifuge tubes maintained at 30 °C for 2 h under a stream of nitrogen gas. Proteolytic activity was expressed as the μmol AA (glycine equivalent) released per h.

5.24 Effects of PCMB on orchard grass protease activity and the reactivity of thiol groups

The effects of binding (masking) thiol groups and protease activity were assessed by adding graded levels of PCMB to an orchard grass protein extract solution. The following procedure was used. To 0.25 ml of the extract prepared as indicated above was added 0.25 ml of either 0.2 M phosphate buffer (pH 6.0) or PCMB stock solutions of varying concentrations and 0.5 ml of 4 % casein. The final concentration of the inhibitor in the assay mixture was 0.0, 0.1, 0.2, 0.3, 0.5, 1.0 and 1.5 mM. After incubating the mixture under a stream of nitrogen at 30 °C for 1 h, 0.5 ml of the reaction mixture was pipetted into a 5 ml test tube and 1 ml of cold 15 % (wt/vol) TCA solution added. The mixture was stored at 4 °C for 1 h and then centrifuged at 10,000 gmax for 10 min. The AA content in the supernatant was assayed using the ninhydrin method indicated above. Amino acids released were expressed as a percent of AA released from the control treatment (as in section 5.23). Ten ml of diluted dithizone were added to 0.5 ml of the remaining incubation mixture for SH assay as outlined by Sasago *et al.* (1963). The estimation of SH groups and the preparation of the standard curve followed the same procedure outlined in Chapter 3.

5.25 Statistical analyses

The General Linear Method of SAS statistical package (1985) was used for the data analyses. A completely randomized design was employed. For tests on the dependence of the proteolytic effect on pH and the effects of chemical inhibitors, the following statistical model was employed;

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

where, μ = overall mean

T_i = treatment (pH, inhibitor) effect

e_{ij} = random error.

The same statistical model used for protease activation and thiol reactivity in Chapter 3, was employed in the analyses of data on the reactivity of thiol groups.

5.3 RESULTS

The fermentation process stabilized between 21 and 45 d after ensiling. This is reflected by the insignificant decrease in pH after 21 d (Figure 5.1). That extensive fermentation took place is also evident from the decline in the concentration of water soluble carbohydrates and in the conversion of protein to NPN (Table 5.1). Evidence of the latter process was shown by the significantly ($P<0.05$) lower content of true protein in silage as estimated by both TCA insoluble CP and true protein (Table 5.1).

Table 5.1 Chemical composition of orchard grass and its silage (DM basis)

	Fresh grass	Silage*
Dry matter (%)	20.72 ± 0.52	14.64 ± 0.36
Crude protein (%)	18.38 ± 0.09	18.73 ± 0.79
TCA insol.CP (%)	15.65 ± 0.11	6.49 ± 0.71
True protein (%)	17.50 ± 0.23	7.96 ± 0.43
sol.CHO (mmol)	0.068 ± 0.003	0.053 ± 0.004
Lactic acid (%)	nd	5.41 ± 0.08

± indicates standard deviation; n=3; nd- indicates not determined. *Ensiling period was 45 d.

5.31 Effect of PCMB or PMSF on orchard grass protease activity

The effect of PCMB and PMSF on the protease activity of the ensiled forage is shown in Figure 5.2. In contrast to the control treatment, both inhibitors significantly ($P<0.05$) reduced protease activity. Up to 95 % of the protease activity during the first 5 d of the ensiling was suppressed by the two inhibitors (Fig 5.2). The inhibition capacity of the two inhibitors was very similar.

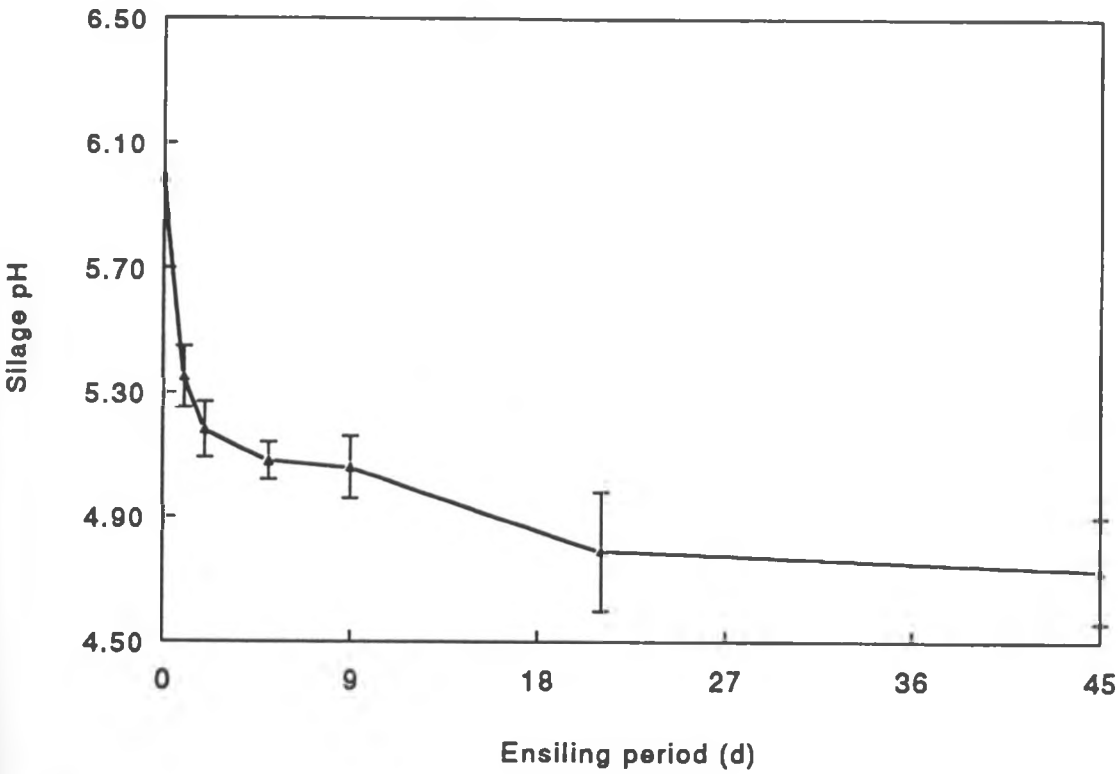


Fig 5.1 Changes in orchard grass pH over the ensiling period

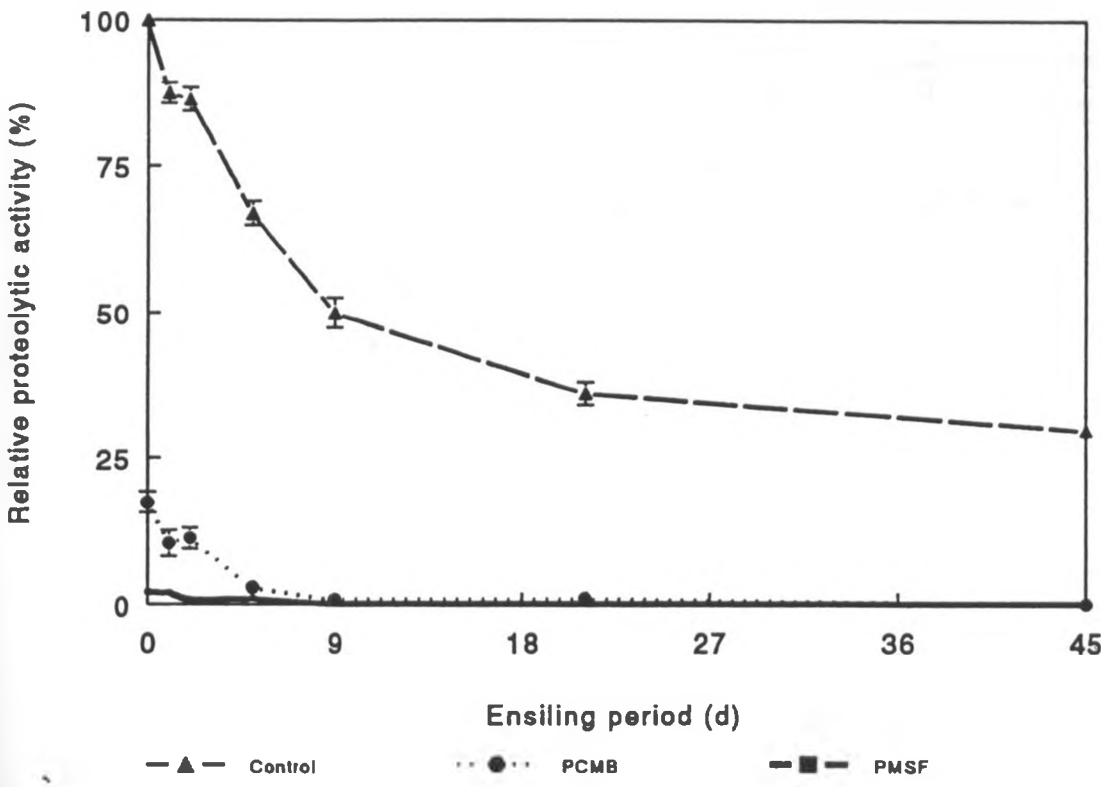


Fig 5.2 Effect of chemical inhibitors on protease activity in orchard grass

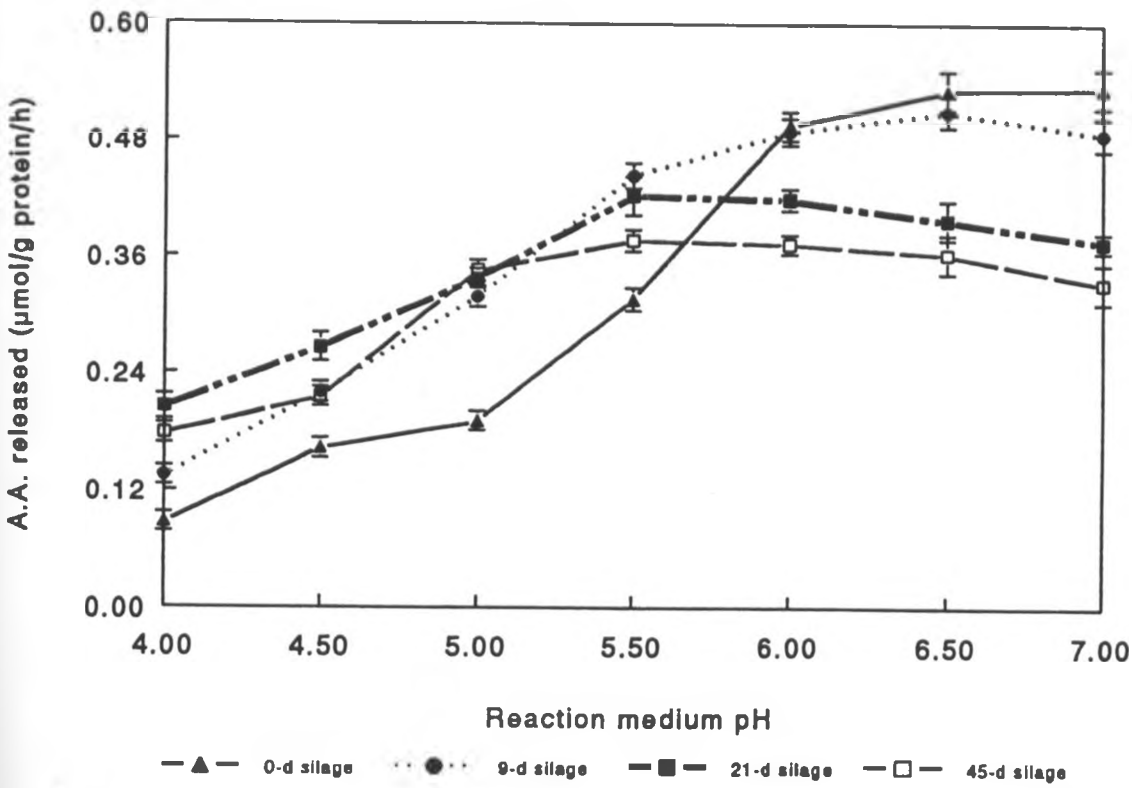
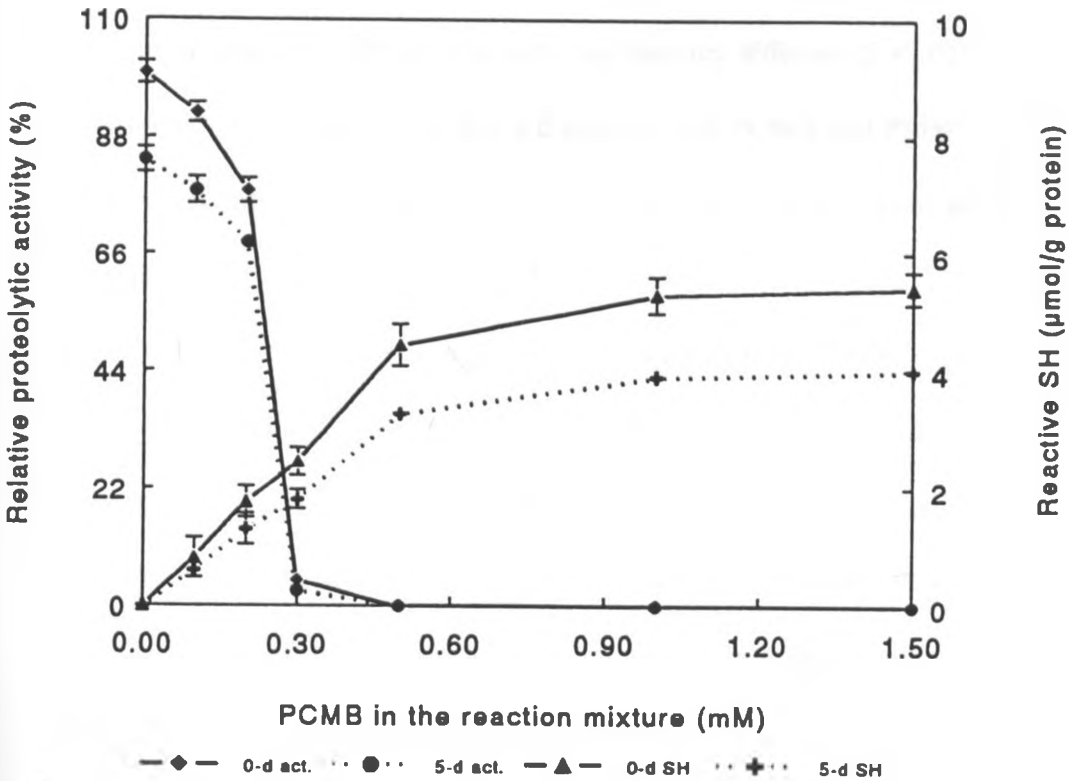


Fig. 5.3 Effect of pH change on the activity of orchard grass proteases



Abbreviation: act=proteolytic activity; 0-d= 0-day ensiling; 5-d=5-d ensiling

Fig. 5.4 Effect of binding reactive -SH groups on proteolysis in orchard grass

5.32 Effect of pH change on orchard grass protease activity

From both Figures 5.1 and 5.3, it was observed that protease activity declined with acidity and the ensiling period. Fig. 5.3 also shows that the optimal pH for proteases in fresh forage was close to neutral pH (pH 6.0 to 7.0). As ensilng progressed, the optimal pH for the proteases was slightly more acidic (pH 5.0 to 6.0). Proteolysis of fresh forage to 5 d ensiling estimated by regression analyses on the control data (Fig 5.2) was 0.70 ± 0.03 compared to 0.38 ± 0.07 (μmol/g/h) between 9 and 45 d post-ensiling. These rates were significantly different ($P < .05$). Up to 100 % proteolytic inhibition occurred after 9 d ensiling with PCMB and PMSF (Fig 5.2). In all cases the rate of decline of protease activity was greater at pH values less than 5.0 than at the pH levels greater than 6.0 (Fig 5.3).

5.33 Effect of PCMB on protease activity and thiol reactivity

From Figure 5.4, it is evident that binding of the CYS-SH groups, through their reaction with PCMB, completely inhibited the protease activity of fresh forage and silage (at 0 and 5 d post ensiling, Fig 5.4). It was also observed that not all the CYS-SH groups needed to be masked in order to significantly quench the protease activity. At 0.3 mM PCMB, over 90 % of the protease activity had been quenched but only half of the CYS-SH groups had been masked by their reaction with PCMB.

5.4 DISCUSSION

The fact that normal fermentation took place is indicated by the significant decrease in water soluble carbohydrates, the reduction in pH and the rapid conversion of protein to NPN. The ensiling of immature grass at high moisture content leads to an average loss of 6 % of the total DM as effluent (Fisher *et al.*, 1981). Obviously effluent losses have a desiccating and a nutrient depleting effect on the resulting silage. Our silage was made in air-tight plastic pails without the possibility of effluent loss. Table 5.1 shows a significant increase in the water content on ensiling. Some of the moisture could have been the product of respiration. Also, during the oven determination of DM, the organic acids could have volatilized with a consequent reduction in the DM content.

Extensive protein breakdown occurs during the first few days when crops are ensiled directly after harvesting. These changes are attributable to plant enzymes whose optimum pH is 5.0 to 6.0 (Ohshima *et al.*, 1979). Our results showed that orchard grass protease activity was completely quenched by PCMB. The pH optima of proteases from fresh forage and the subsequent silage were 6.0 - 7.0 and 5.0 - 6.0 respectively (Fig 5.3). Zucker *et al.* (1985) indicated that papain and related plant CYS-SH proteases generally show maximal activity on synthetic substrates and protein at neutral or slightly alkaline pH. This is possibly what is observed with fresh plant proteases. However, on ensiling the intracellular compartmentalization is lost either due to mechanical disruption during harvest or as a result of cellular disintegration under anaerobic conditions. Consequently, lysosomal proteases are released.

Lysosomal CYS-SH proteases are known to be unstable at alkaline pH levels (Barrett, 1976). Certainly this contributes to the fact that in most assay systems they have acidic pH optima. McKersie (1981) has postulated a succession of substrate specific proteases with varying pH optima when alfalfa herbage was ensiled. However, in this study proteolytic activity was maximal at close to neutral pH, declining with the ensiling period and increased acidity (Fig. 5.2 & 5.3). McKersie (1985) also made similar observations. Other evidence indicates that under favourable conditions, assays with synthetic substrates can reveal pH optima above neutral pH (Willenbrock and Brocklehurst, 1984; Mason *et al.*, 1985). This suggests that the catalytic mechanisms of lysosomal CYS-SH proteases may not be fundamentally different from those of the plant enzymes. Hence, as established in Fig. 5.2 and also by McKersie (1981), plant proteases would be susceptible to mercaptidation (the reaction of -SH groups with mercury compounds, e.g. PCMB). Assays with protein substrates tend to show low pH optima for the additional reason that the substrate becomes unfolded and more susceptible to hydrolysis under acidic conditions.

Low pH (< 5.0) was observed to result in reduced proteolysis. Besides substrate unavailability and end product inhibition, pH is thought to regulate the activity of plant proteases (Papadopoulos and McKersie, 1983; McKersie, 1981; 1985; McKersie and Buchanan-Smith, 1982). In our study on the effect of pH (Fig. 5.3), casein substrate was included coupled to a short incubation period (2 h). Thus, it is unlikely that substrate deprivation or end product inhibition would have had such a significant reduction on the protease activity. CYS-SH proteases are known to contain

a catalytically active CYS-SH and a histidine imidazole group (His-159) within the active site of group (Cys-25) of the enzyme (Rich, 1986). However, thiol groups take part in most reactions as mercaptide (thiolate) ions, RS^-. Hence, to facilitate catalysis the imidazole group is understood to polarize and deprotonate the Cys-25 SH group forming a highly nucleophilic ion pair. Consistent with this reaction mechanism is the fact that the CYS-SH group behaves like a weak acid and its deprotonation would be less favoured at low pH. In fact Elson and Edsall (1962) established the pH dependence in the intensity (ionization) of the SH bond of cysteine from the Raman spectrum. It is possible that the active centres are adequately ionized at the pH optima noted above and an in situ increase in acidity would compromise their dissociation and ultimately limit net catalysis.

Rich (1986) represented the reaction mechanism of cysteine proteases by the following pathway; $E + S \Leftrightarrow ES \Leftrightarrow ES' + P^1 \Rightarrow E + P^2$

(E, enzyme; S, substrate; ES, enzyme-substrate complex; P^1 & P^2, product 1 and 2 respectively). In step three of the reaction mechanism, the acyl group of thio ester, (corresponding to ES'), is transferred to a water molecule in a slow process to liberate the carboxylic acid and regenerate the active enzyme. Attack of water on the thioester in the deacylation step is catalyzed by the imidazole group of His-159. Consequently, low pH or very rapid moisture reduction, e.g. on wilting, would have a lower protein breakdown. In the conventional silage making practices these factors are employed with varying measures of success.

That ensiling resulted in a lower CYS-SH content and reduced proteolytic activity (Fig. 5.4), could be attributed to thiol oxidation in the catalytic centres. At optimal physiological function, glutathione (GSH) protects the oxidation of the active sites (Rennenberg, 1982). At 0.3 mmol PCMB (Fig. 5.4), proteolytic activity was almost totally suppressed, yet almost 25 % the CYS-SH content remained unmasked by PCMB. This shows that not all the CYS-SH groups in the protein extract were involved in catalysis. As observed by Kawashima and Wildman (1970), the non-catalytic CYS-SH groups in the protein could be merely stabilizing the structural configuration. Conversely, rapid suppression of the catalytically active CYS-SH groups may be more effective in ensiling techniques that aim for superior results in controlling proteolysis.

5.5 CONCLUSIONS

Although no effluent losses were possible, ensiling orchard grass in air-tight plastic pails resulted in extensive breakdown of the protein to NPN. It was found that the pH optima for protease in the fresh herbage and silage were 5.5 - 6.5 and 5.0 - 6.0, respectively. Protease activity declined with both the ensiling period and increased acidity. At lower than pH 5.0, protease activity dropped to below 40 % of the optimal activity. Inhibitor studies with PCMB and PMSF elucidated the involvement of thiols in the protease activity. However, not more than 75 % of the CYS-SH content (about 0.2 - 0.3 μmol SH/g true protein, was involved in catalysis. The rest of the CYS-SH groups in the protein configuration could be providing structural stability. Based on the catalytic mechanism of cysteine proteases, low moisture content and decreased pH

would reduce the proteolytic activity. With wet orchard grass, investigations are needed to discern whether cysteinyl catalytic inhibitors could be usefully incorporated during ensiling. To the extent that proteases in orchard grass were shown to be cysteinyl in nature, SH reagents would be expected to manipulate their proteolytic activity. This premise was investigated in the subsequent study.

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Chapter 6

MODIFICATION OF SILAGE FERMENTATION IN ORCHARD GRASS

6.0 ABSTRACT

The objective of this study was to evaluate the effects of common surfactants and thiol-oxidants on the fermentation of orchard grass silage. Immature orchard grass (50 kg) harvested with a forage harvester was immediately sprayed with one of the following additives and ensiled at 30 °C for up to 45 d (concentration of additives was calculated per litre of fresh forage homogenate): (a) no additive, (b) 0.3 % Teepol, (c) 0.3 % Teepol + 8.0 mM cysteine, (d) 10.0 mM potassium iodate, (e) 10.0 mM potassium iodate + 8.0 mM cysteine, (f) 0.3 % SDS, (g) 0.3 % SDS + 8.0 mM cysteine, and (h) 0.5 % Tween-80. Results showed that extensive proteolysis occurred in the absence of any of the additives. With Tween-80, rapid proliferation of lactic acid producing bacteria could have resulted in the significantly ($P < .05$) lower final pH (pH 4.4 compared to 4.7 in the control silage). The rapid drop in pH was believed to have prevented extensive protein breakdown. Treatments with SDS and Teepol were associated with increased masking of protease -SH functional groups. Their reactivity was significantly reduced. Consequently, the TCA-precipitable CP content increased significantly with these treatments. Extensive thiol oxidation coupled to sharp inactivation of silage proteases occurred in the presence of potassium iodate. However, addition of cysteine increased the silage protein fermentation. From these results it is

evident that potassium iodate and anionic surfactants could be used to suppress silage proteolysis.

(**Keywords**: orchard grass, silage, surfactant, oxidant, thiol)

6.1 INTRODUCTION

On ensiling, plant proteases embark on vigorous catalytic processes which reduce the nutritional quality of silage. Proteins in ensiled herbage are converted to amino acids, peptides, and other forms of non protein nitrogen (Ohshima *et al.*, 1979). This compromises the quality of nutrients in silage-based rations, particularly in meeting the metabolizable protein requirements of lactating dairy cows. Considerable research effort has strived to enhance silage nutrient preservation, especially in extremely wet regions where wilting may not always be practicable. Muck (1988) reviewed factors that influenced silage quality and their management implications. Numerous workers agree that controlling proteolysis is paramount in improving silage quality. Since maximum protease activity occurs at close to neutral pH, (McKersie, 1981; 1985), some researchers have advocated lowering pH with organic acids. Others prefer silage inoculants to reduce the pH (Kung *et al.*, 1991).

Plant proteases are released during ensiling, either as a result of mechanical disruption during harvesting or cellular disintegration under anaerobiosis. On release, proteases could either interact with their protein substrate or chemical additives designed to slow them down. The importance of cysteine (CYS-SH) proteases in orchard grass silage fermentation was discussed in Chapter 5. McKersie (1981) also postulated that p-chloromercuribenzoate (PCMB), an -SH inhibitor, could be used against the neutral proteases and aminopeptidases to control proteolysis. Consequently, the design of additives which regulate silage protein breakdown may require some insight into CYS-SH proteases and their reaction chemistry.

The -SH groups in most cysteine proteases participate in chemical reactions as RS^- (Rich, 1986) or free thiols (-SH) (Klein and Kirsch, 1969 a,b). The latter have also related the reactivity of -SH groups to the protease activity. Available evidence shows that -SH groups of most proteases are located in hydrophobic environments within the enzyme molecules. A variety of factors could influence the reactivity of these groups within protein macromolecules. An important factor affecting the ionization (reactivity) of -SH groups in protein molecules is the electrostatic influence of its charged neighbours. Heitmann (1968) found that the incorporation of -SH groups into anionic micelles leads to their "masking", i.e. a sharp decrease in their reactivity. In addition, unionized -SH groups of proteins in these environments could be masked further by hydrophobic interactions.

Like orchard grass proteins, wheat gluten contains a high proportion of thiols that are closely associated with dough properties. Nonionic surfactants, e.g. Tween-80, are used widely as wetting, dough strengthening, and gluten extending aids (Stutz, *et al.*, 1973). The resultant dough is more tolerant to mechanical abuse, (kneading), and the bread has superior volume and texture. Tween-80 has also been used to increase cellulase activity (Castanon and Wilke, 1981). Oxidizing agents, e.g. potassium iodate and ascorbic acid, are popularly used as dough improvers (Tsen, 1964; Tsen, 1965; Stauffer, 1983). The latter processes involve the enhancement of disulfide formation via thiol-disulfide interchange (Hird and Yates, 1961; Tsen and Bushuk, 1963; Tsen, 1966) to build up a gluten "super molecule" (Stauffer, 1983). The corollary to the foregoing is that oxidizing agents and common surfactants could aid in manipulating the reactivity

of -SH groups in orchard grass proteases. Hence, the objective of this study was to evaluate the effects of common surfactants and thiol-oxidants on orchard grass silage fermentation.

6.20 MATERIALS AND METHODS

6.21 Experiment 1. Silage preparation

Immature orchard grass was harvested (New Holland, Forage Harvester) from Agriculture Canada field plots, at Agassiz, B.C. Fifty kg of the forage was thinly spread out on plastic mats and sprayed with a surfactant additive dissolved in water (to 500 ml final volume) and the forage was tightly packed into air-tight (2.5 kg capacity) plastic pails. Additive application and packing into the pails took approximately 1 h. Approximately 2.0 kg was packed per pail. The anionic surfactant levels were chosen on the basis that 0.3% of Teepol was found to denature ruminal digesta proteins (Chapter 4). The level of 0.5% Tween 80 was found to provide maximal proteolytic activity (Chapter 3). Hence, the chosen treatments were;

a) no additive,

b) 125 ml Teepol (Tp),

c) 125 ml Tp + 6.33 g cysteine (CYS),

d) 80 g KIO_3,

e) 125 g sodium dodecylsulfate (SDS),

f) 125 g SDS + 6.33 g CYS,

g) 80 g potassium iodate (KIO_3) + 6.33 g CYS, and

h) 200 ml Tween 80.

After spraying, the forage was ensiled and stored in a room maintained at a constant 30 °C for either 0, 1, 2, 5, 9, 21, and 45 d. Each treatment was replicated three times. At the end of each ensiling period, the silage was mixed and subsampled

(500 g). A final 200 g sample was obtained and ground under liquid nitrogen (-196 °C) using a coffee grinder. The samples were stored at -50 °C pending further analyses. The subsequent analyses were dry matter (DM), crude protein (CP), trichloroacetic acid insoluble crude protein (TCAppt-CP), lactic acid, pH, water soluble carbohydrates (CHO). The methods used were outlined in Chapter 5.

6.22 Experiment 2. Effect of additives on orchard grass protease activity and reactive thiol groups

The following was the protocol used to determine the effect of Teepol, SDS, Tween 80 and KIO_3 on the proteolytic activity and thiol reactivity in orchard grass protein extracts. Fifty grams of the control fresh forage were fractionated as indicated in Chapter 5. An extract solution was obtained by resuspending 5 g of the orchard grass extract obtained as described in Chapter 5 in 100 ml of 0.2 M phosphate buffer, pH 6.0 containing sodium isoascorbate (10mM). One ml of the extract was incubated with an equal volume of 4 % casein, 2 ml of the appropriate additive stock solution and 1 ml of buffer solution at 30 °C for 2 h under a stream of nitrogen gas. At this temperature proteolysis is supposed to be at its maximum rate. A final 4 ml total volume and surfactant concentrations 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, and 1.0 % in the assay mixture were maintained. In the case of KIO_3 the final concentrations in the reaction mixture were 0, 0.25, 0.5, 1, 2, 5, and 10 μM. Two ml of the assay mixture were removed and used in the determination of -SH and disulfide (-SS) content using the procedure of Sasago et al. (1963) outlined in Chapter 4. The reaction in the remaining 2 ml was stopped by the addition of 2.0 ml of cold (4 °C) 20 % (wt/vol),

TCA solution and in an ice bath for 1 h. After cooling, the precipitate was removed by centrifugation (10,000 gmax for 10 min.). Free amino acids were estimated in 0.1 ml of the supernatant by the ninhydrin method (Rosen, 1957). Activities were expressed as a percentage of amino acids (glycine equivalent) released μmol/g/hr relative to the control.

Reagents for the -SH and -SS determination and the subsequent assays were carried out as outlined in Chapter 4. The decline in reactive -SH content with increase in the surfactant concentration in the reaction mixture was referred to as -SH masking. The opposite effect was referred to as -SH unmasking. In the relevant cases, the decline in relative proteolytic activity were attributed to -SH masking and vice versa.

6.23 Statistical analyses

The data were analyzed as a factorial experiment in a completely randomized design. Analysis of variance was performed by PROC GLM of the SAS Statistical Package (1985). Means were compared using Tukey's method.

The model for a completely randomized design (Chapter 5) was used in the statistical analyses of the silage composition data. Similar statistical models as used with protease activation and thiol reactivity data in Chapter 3, were employed in the analyses of data from the protease activity, and thiol and disulfide reactivity.

6.30 RESULTS

6.31 Experiment 1. Effects of additives on silage composition

The calculated rate of additive incorporation based on the fact that the dry matter content of fresh orchard grass was 20.7 % is shown in Table 6.1. The values are provided on the basis of a fresh homogenate as these would be the additive concentrations once cellular compartmentalization is lost (during the ensiling process). Furthermore, these would be the concentrations of the additives in the reaction (aqueous) media that cellular reactions take place in.

Table 6.1. Additive concentration during ensiling

Additive	conc. fresh homogenate
SDS	0.3 %
Teepol	0.3 %
Tween-80	0.5 %
Cysteine	8.0 mM
KIO_3	10.0 mM

The chemical composition of the ensiled immature prebloom orchard grass is given in Table 6.2. The ADF content of the fresh material was 35.2 %.

As shown in Figures 6.1 and 6.2, the pH dropped during the ensiling process in all treatments. The pH after 45 d ensiling was highest with the anionic surfactant- and KIO_3- based additives lowest with Tween-80. The pH of the control treatment was intermediate between that of the anionic and that of Tween 80. In all treatments, the pH values seemed to stabilize after 21 days of ensiling.

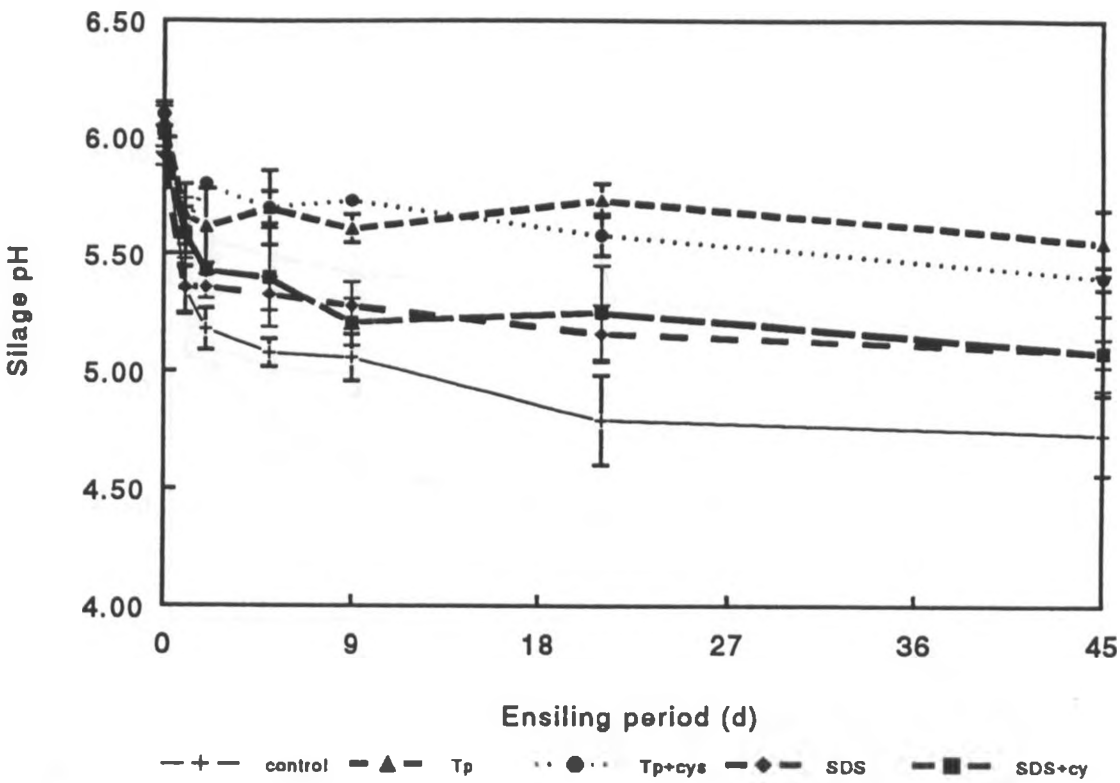


Fig 6.1 Effect of cysteine, Teepol and SDS on orchard grass silage pH

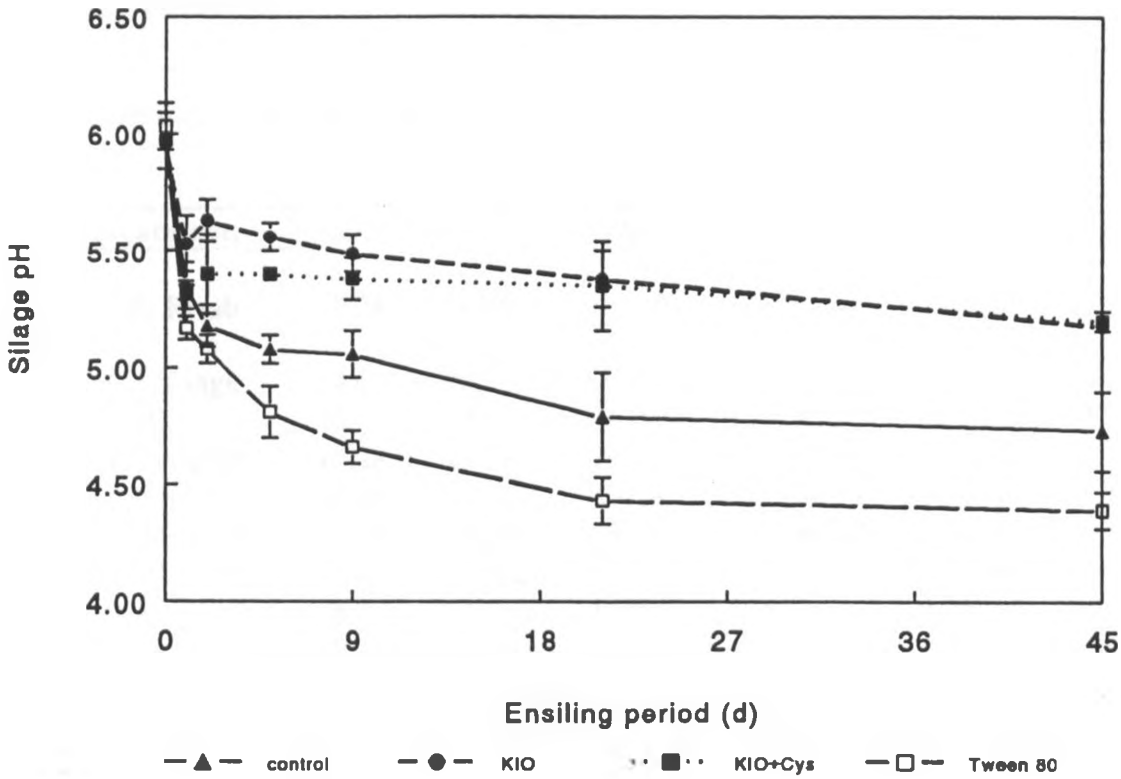


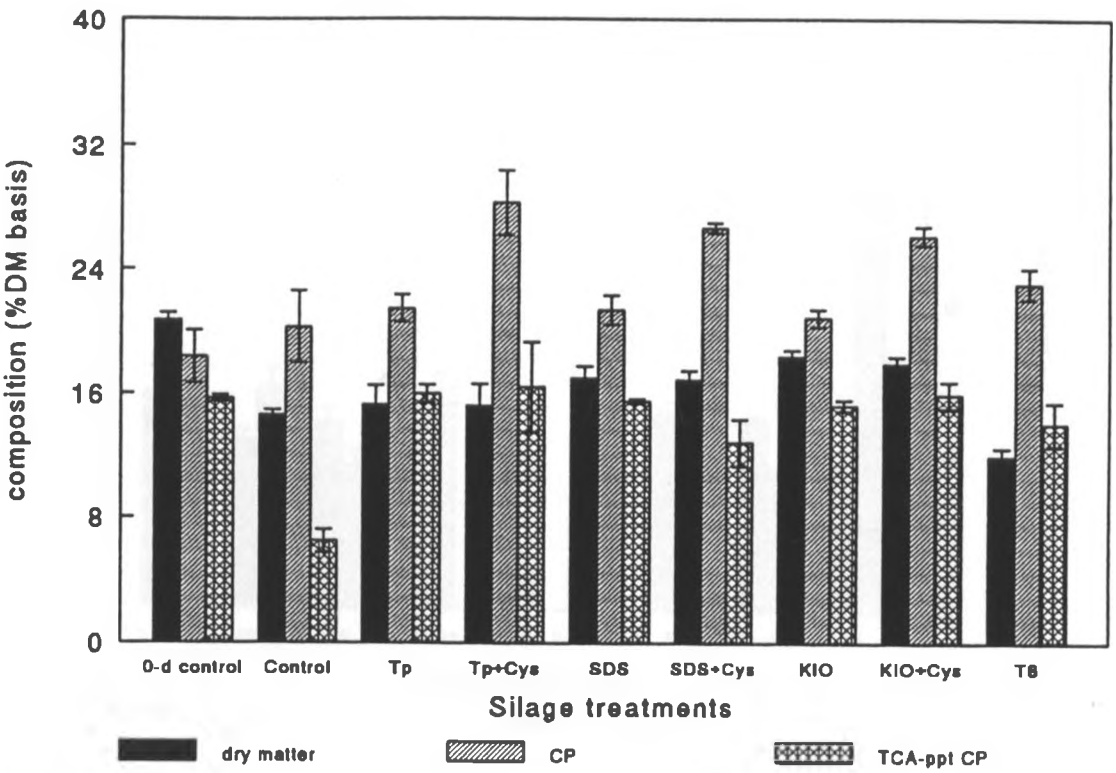
Fig. 6.2. Effects of cysteine, Tween 80 and potassium iodate on orchard grass silage pH

The effect of the treatments on silage composition shown in Table 6.2 were compared in Figures 6.3 and 6.4. In comparison with fresh orchard grass, all treatments (except with KIO_3) underwent extensive fermentation as evidenced by the decline ($P < .05$) in both water soluble carbohydrates and pH and, a rise in the lactic acid content. In addition, the DM content declined, particularly in the control and Tween-80 treated silages. Silage protein fermentation was influenced by additives as indicated by the significant ($P < .05$) increase in TCA precipitable protein of all treatments compared with the control.

Table 6.2. Chemical composition of fresh and ensiled (45 d) orchard grass (DM basis).

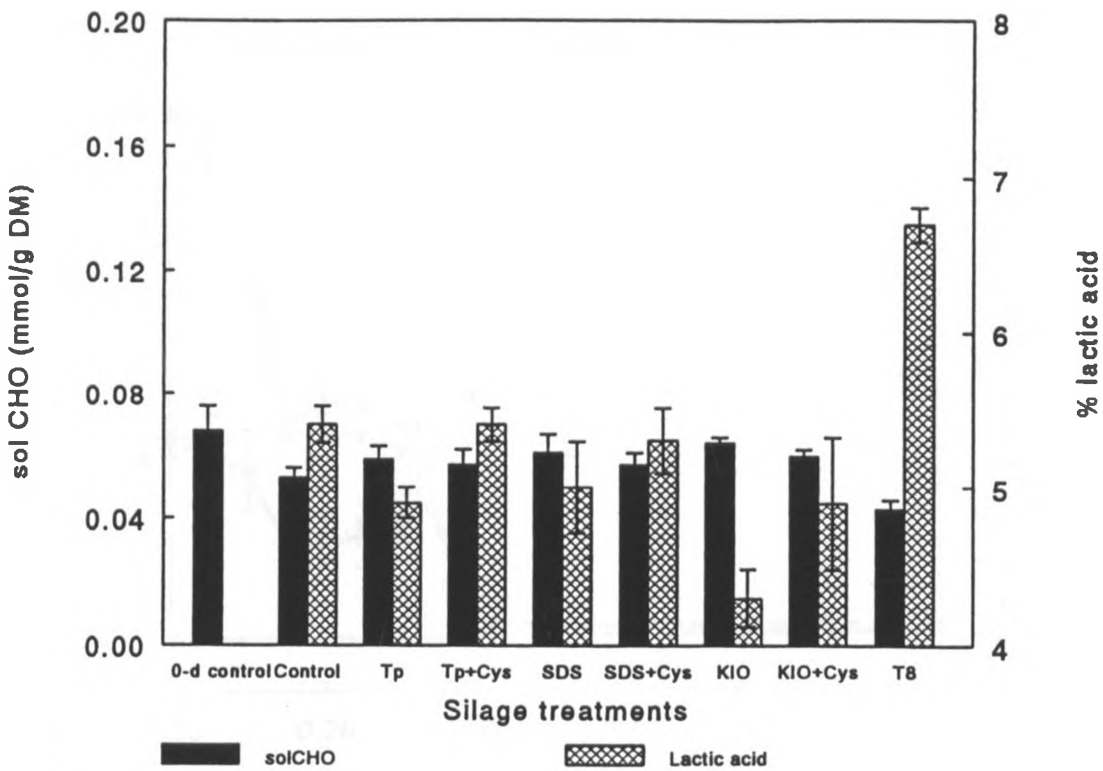
Treatment;	%DM	%CP	%ppt. CP	%Lactic acid	[1]sol. CHO	pH
Ctl. Fresh	20.7a	18.4d	15.7ab	nd.	0.068a	5.9a
Ctl. silage	14.6e	20.3cd	6.5d	5.4b	0.053d	4.7e
TPL	15.3e	21.5cd	16.0ab	4.9bc	0.059bcd	5.6b
TPL+CYS	15.2e	28.3a	16.4a	5.4b	0.057cd	5.4bc
KIO	18.4b	20.9cd	15.2ab	4.3c	0.064ab	5.2cd
KIO+CYS	17.9bc	26.2ab	15.9ab	4.9bc	0.060bcd	5.1d
SDS	17.0cd	21.4cd	15.5ab	5.0bc	0.061bc	5.2cd
SDS+CYS	16.9d	26.7ab	12.8b	5.3b	0.057cd	5.1d
Tween 80	11.9f	23.1bc	14.0ab	6.7a	0.043e	4.4f

Values within a column followed by different letters differ ($P < .05$). Ctl. = control; nd. = determined; %ppt.CP = % TCA precipitable crude protein; sol.CHO = soluble carbohydrates
[1] units mmol/gDM (glucose equivalent). n=3.



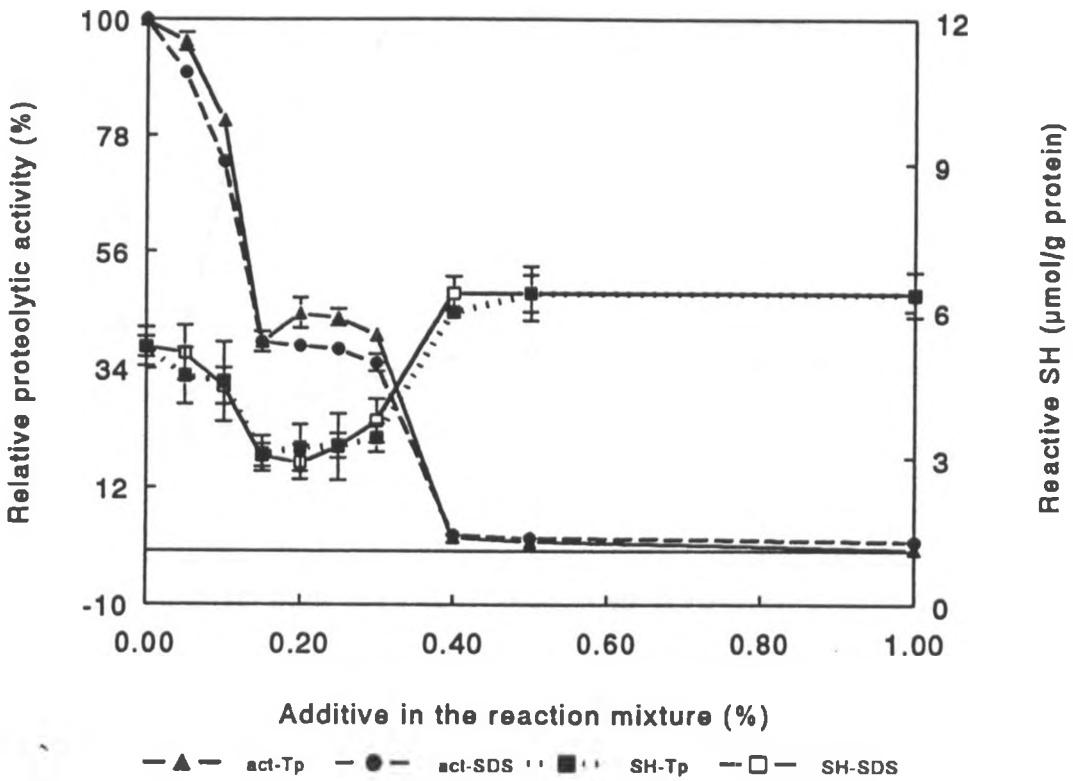
Abbreviations: 0-d = fresh orchard grass; 45-d = 45 day silage; Tp = Teepol; T8 = Tween 80

Fig. 6.3. Dry matter and protein composition of orchard grass silage



Units: sol CHO = mmol/gDM; lactic acid = %DM

Fig. 6.4. Lactic acid and soluble carbohydrate in orchard grass silage



Abbreviations: act=proteolytic activity; Tp=Teepol HB7

Fig. 6.5. Effect of Teepol HB7 and SDS on protease activity and thiol reactivity

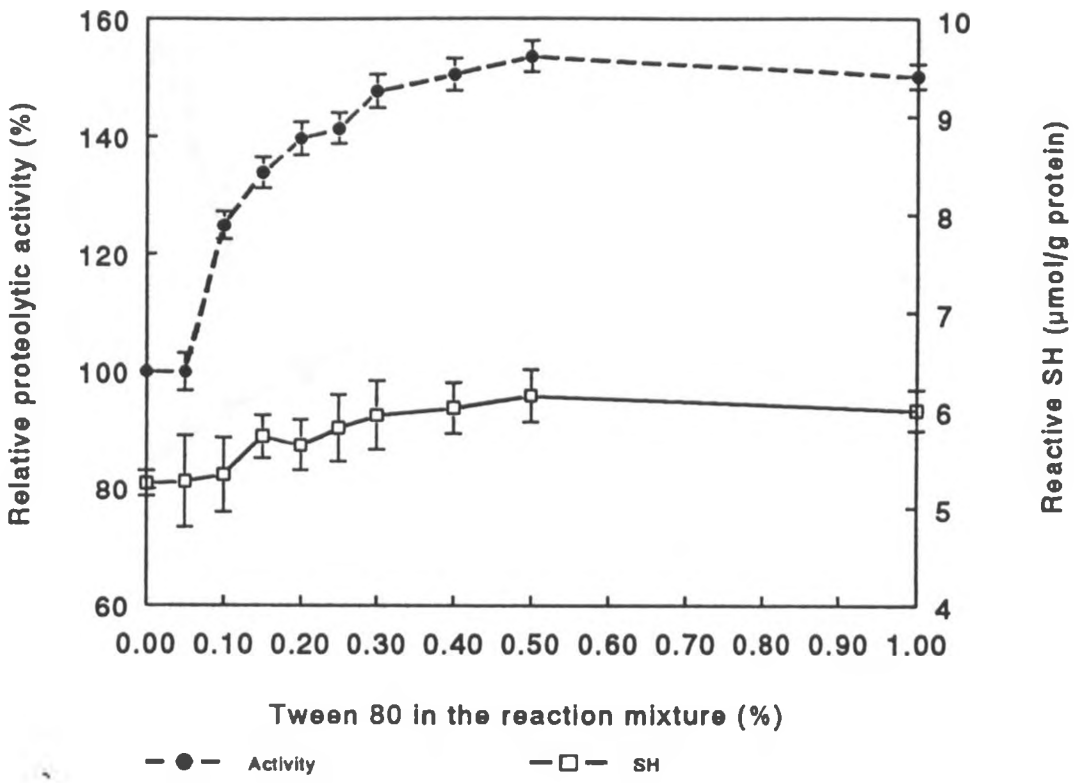


Fig. 6.6. Effect of Tween 80 on the protease activity and -SH reactivity in orchard grass

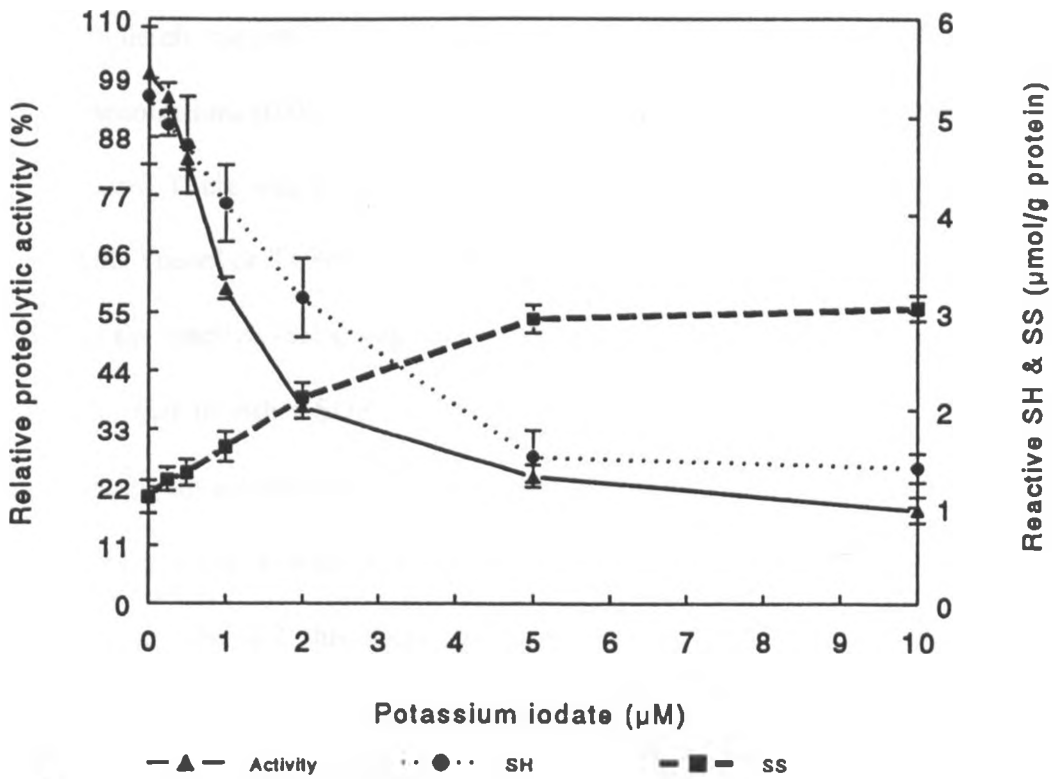


Fig. 6.7. Effect of potassium iodate on protease activity, -SH and -SS reactivity

6.32 Effect of additives on orchard grass protease activity

A mechanism through which these additives could have mediated their activity is shown in Figures 6.5, 6.6 and 6.7. These figures show the change in protease activity and the reactivity of thiols with various levels of surfactants. Parallel measurements of proteolytic activity and the reactive (accessible) -SH content were made. The addition of either SDS or Teepol reduced the activity of silage proteases. However, not until the surfactant concentration was greater than 0.4 % was proteolysis completely suppressed and all the accessible -SH groups unveiled. SDS and Teepol concentrations (0.05 to 0.3 %) significantly ($P < .05$) masked the reactivity of the -SH groups. There was no significant ($P < .05$) change in the disulfide content with either SDS, Teepol or Tween-80 treatments. The parallel response of both proteolytic activity and the reactive -SH groups to the addition of Tween-80 differed significantly ($P < .05$) from that of either SDS or Teepol. The addition of up to 0.5 % Tween-80 increased proteolytic activity and the proportion of reactive -SH groups ($P < .05$) with orchard grass extracts. Levels of Tween-80 above 0.5 % did not enhance this response ($P > .05$). In Table 6.2, this effect was not established, as the true protein content was higher ($P < .05$) than that of the control. Clearly, this was in contradiction to our expectations.

The effect of potassium iodate on the parallel measurements of proteolytic activity and reactive -SH groups is shown in Fig. 6.7. Unlike the surfactant treatments mentioned above, 0.25 to 5 μM KIO_3 increased the accessible disulfide content by approximately half the rate of decline of the reactive thiols content. Proteolytic activity also declined ($P < .05$) within the same range.

6.4 DISCUSSION

The pH declined from an initial average value of 6.0 over the ensiling period for all treatments. The extent of the drop however varied with different treatments. Lactic acid bacteria have been associated with increased acidity on ensiling. McDonald (1981) observed that lactic acid bacteria proliferate rapidly on released plant juices, (particularly the soluble carbohydrates), producing lactic and acetic acids which result in the pH changes commonly observed in silages. Table 6.2 shows a significant decline in water soluble carbohydrates while lactic acid increased in all silages. The decline in water soluble carbohydrate could be the result of rapid respiration that may have occurred at the high ensiling temperature (30 °C). The two important products of cell respiration are water and carbon dioxide. In the systems used here, resulting effluent was retained in the plastic silo. However, in ordinary silos involving high moisture grass the effluent would be about 6 % of the DM (Fisher *et al.*, 1981). These sources of water could have contributed to the increase in moisture content on ensiling that was observed across all the treatments including the control (Table 6.2). In lactic acid fermentation, 1 glucose $\Rightarrow$ 2 lactate + H_2O. However H_2O is used when each glucose is cleaved from starch. Hence, the net H_2O production is zero. If the system were aerobic then water and carbon dioxide would be formed. The growth of mould in the silage was not extensive. Although mould growth reflects aerobic fermentation, the low pH values obtained, particularly with the Tween 80 treatment, indicates that lactic acid fermentation predominated.

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In comparison with the control, less silage fermentation occurred with SDS and Teepol. This is shown by the higher ($P < .05$) pH and water soluble carbohydrate content and lower lactic acid content. In addition, proteolysis was suppressed ($P < .05$) with these two treatments. This was evidenced by the higher ($P < .05$) proportions of TCA-precipitable proteins (true proteins) assayed. Protease activity estimates (Figure 6.5-6.7) also showed extensive proteolytic inhibition, as the masking of -SH by up to 0.3 % SDS or Teepol progressed (Figure 6.5). At high concentrations, the two anionic surfactants have strong denaturing ability. The involvement of cysteine proteases in silage protein breakdown has been discussed in Chapter 5 and by McKersie (1981). Further, Csoma and Polgar (1984) established a similar mechanism in the protease from germinating bean cotyledons. The -SH groups of cysteine proteases, including papain, are usually located in hydrophobic environments. Heitmann (1968) found that incorporating -SH groups into anionic micelles leads to a sharp decrease in their reactivity. However, Figure 6.5 showed that the levels of reactive (accessible) protease -SH groups were highly correlated with proteolytic activity up to 0.25 % SDS or Teepol. These results concur with postulates by Klein and Kirsch (1969 a,b) who also observed that the activity of papain preparations is directly proportional to the content of free -SH groups. Further, they showed that the inactive form of papain is a mixed disulfide formed by the -SH group of the protein and free cysteine. The masking of -SH groups was also observed in milk by Sasago *et al.* (1963) when SDS was added at 0.25 %. In baking, low levels of SDS (<0.5 %) were found to increase the dough mixing time and was attributed to the masking of the -SH groups of gluten (Danno and

Hoseney, 1982). Higher than 1 % SDS gave a shorter dough mixing time, a fact attributed to unmasking of -SH groups and to thiol disulfide interchange. These results concur with our findings (Figure 6.5) that low levels of either of the anionic surfactants resulted in masking of -SH groups (reduced -SH reactivity) while extensive unmasking of -SH and protein denaturation occurred at levels higher than 0.4 %. Results from our parallel determination of -SS showed no significant effects.

Addition of 8 mM cysteine to the anionic surfactants increased silage fermentation rates significantly ($P < .05$). However the addition of cysteine also contributed to the high CP values observed. That cysteine may participate in the reduction of -SS bonds and in the disulfide exchange is a well known phenomenon. Living cells contain considerable amounts of low molecular weight thiols including glutathione. The latter protects the -SH groups of intracellular enzymes from oxidation and is involved in the removal of free radicals and peroxides (Rennenberg, 1982).

The inclusion of Tween-80 in the silage increased the fermentation rate as shown by the significantly lower pH and higher lactic acid concentration (Table 6.2) than measured in the other treatments. Tween-80, a nonionic surfactant has been found to increase cellulase activity in anaerobic digesters, (Castanon and Wilke, 1981; Madamwar *et al.*, 1991), and lipase activity (Hazlewood and Dawson, 1975) in in vitro rumen media. Increased viable microbial colony counts have been observed in Tween-80 treated ruminal contents (White *et al.*, 1988). That the content of reactive -SH also increased with Tween-80 treatment (Figure 6.6) may point to increased silage protein breakdown. However, the high fermentation rates may also have resulted in extensive

proliferation of a silage microbial biomass. Consequently, the significant increase in TCA-precipitable protein observed may have been of microbial origin. Alternatively, the extensive proteolysis which normally occurs in the early stages of ensiling (at around neutral pH), may have been prevented by the rapid establishment of lactic acid producing bacteria. It is known that the aerobic silo environment that temporarily exists immediately after ensiling results in hydrogen peroxide production by lactic acid bacteria since oxygen is used as a terminal acceptor of electrons (McDonald, 1981). Peroxide impurities in Tween-80 may have contributed some of the peroxide effects over this period. The hydrogen peroxide could have inactivated the degradative protease -SH functional groups (Herriott, 1947; Neumann, 1972). In turn the hydrogen peroxide could have been decomposed to water and oxygen by catalase (Lehninger, 1982) or further attacked the unsaturated fatty acids of membrane lipids. The former would contribute to the decrease in silage dry matter observed. The loss of cellular compartmentalization due to the degradation of membrane lipids may have contributed to rapid establishment of lactic acid bacteria and the observed rapid increase in silage acidity. Evidence also exists that Tween-80 could protect enzymes against damage due to shear and surface denaturation (Reese, 1980). The low pH would further reduce proteolysis via reduced ionization of the protease -SH groups.

Rapid proteolysis due to Tween 80 could also have been the consequence of accelerated ethylene production. Ethylene regulates many aspects of plant growth, development and senescence (Yang and Hoffman, 1984). These researchers noted that ethylene could be derived from lipid peroxidation. They associated ethylene produced

by lipid peroxidation to the ancient cultural practice of hastening fig growth and maturation by the application of olive oil to the fruits. The Tween 80 used had 75% oleic acid and a small amount of peroxides as impurities.

There was a significant oxidation of orchard grass thiols to disulfides as shown in Figure 6.7. Within the range 0.25 to 5 μM of KIO_3, the disulfide content increased at approximately half the rate of decrease in thiol content and, proteolytic activity was also inversely related to the accumulation of disulfides (Figure 6.7). This could have contributed to the significant suppression of silage proteolytic activity noted in Figures 6.7 and Table 6.2. The effect of KIO_3 on thiol oxidation is well known in baking (Hird and Yates, 1961; Tsen and Bushuk, 1963; Tsen, 1964; Tsen, 1965 Tsen, 1966; Stauffer, 1983). Together with cellular glutathione and low molecular weight protein thiols, the addition of 8 mM cysteine may have reduced the effect of KIO_3. Thus silage fermentation increased when free cysteine was added to KIO_3. Further, water is produced in the process of thiol-disulfide interchange (Hird and Yates, 1961). This could have contributed to a significantly higher silage moisture content compared to the other treatments. However, degradation of water soluble carbohydrates possibly through respiration that would yield water was significantly ($P < .05$) reduced. This probably offset the effect due to thiol-disulfide interchange.

6.5 CONCLUSIONS

The results from these studies showed that extensive protein fermentation occurred on ensiling orchard grass. However this was controlled by either inhibiting protease activity or enhancing lactic acid production. Low levels (< 0.5 %) of anionic

surfactant and potassium iodate (10 μM) based additives were successfully used to slow down proteolysis. In practical terms, accessibility of surfactants to their action sites would need further research. Chopping forage could help expose the intracellular proteases to the additives. As an alternative, Tween-80 which is a nonionic surfactant, was used. The latter accelerated silage fermentation and lowered the pH such that proteolysis was significantly suppressed. A combination of Tween-80 and anionic surfactants or potassium iodate would possibly have an superior additive effect.

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Chapter 7

7.0 GENERAL CONCLUSIONS AND RECOMMENDATIONS

In this study, the role of catalytically active CYS-SH residues in rumen and silage fermentation was investigated. It was established that CYS-SH proteases play a predominant role in the fermentation processes in comparison with other classes of proteases. Furthermore, it was observed that at low levels (0.5%) Tween 80 enhanced rumen and silage fermentation and increased both feed intake and apparent feed digestibility significantly ($P < .05$). On the other hand, 0.1% Teepol reduced proteolytic activity ($P < .05$) and protein degradation without adverse effects on intake and apparent digestibility of the feed. At 10 mM., potassium iodate (a thiol oxidant) reduced proteolysis in silage significantly.

Although, promising as these additives might be, successful manipulation of proteases will be highly dependent on the biochemical properties of their CYS-SH residues. Furthermore, the silage (feed), the rumen microbes, the animal, and the feed formulator, all form an integrated biological system. The proposed additives have to be safe to handle. Not to mention that ingredients proposed in the development of a viable additive have to be cost effective and preferably metabolizable with nutritional benefits. Further, a proposed additive might seem appropriate for instance in silage fermentation while inappropriate in feed rations. In other cases a preservation strategy might involve the sequential introduction of additives of contrasting effects. Hence, successful exploitation of the CYS-SH characteristics may not be completed in one study as was

conducted here. However from this study some conclusions and suggestions could be made.

For the purposes of discussion, this subject will be divided into fermentation activators and inhibitors encompassing thiol unmasking and masking, respectively. It is also important to remember the similarities between the catalytic mechanisms of serine and cysteine proteases. Both have strongly nucleophilic amino acids at their catalytic sites and hence, electrophilic inhibitors would be most successful. In addition, catalytic residues in the two protease classes have been associated with amylolytic activity and glycolysis.

7.1 Activation of fermentation in the rumen

At 0.5% Tween 80 in the diet, feed DM intake was increased by 40% and apparent digestibility of ADF, NDF and CP were enhanced ($P < .05$) by up to 10 percentage points. In vitro tests also showed increased thiol unmasking, microbial protein adsorption and cellulose degradation. Fermentation activation could therefore be divided into; (a) non-nutrient specific, and (b) specific nutrient activation of rumen fermentation.

(a) *Non-nutrient specific activation of rumen fermentation.* Where ruminant production is based on forages including silages, microbial protein synthesis and not bypass protein is the primary concern. Low appetite, nutrient imbalance (both fermentable sugars and amino acids - in silages) and compromised physical capacity of the rumen are usually the major limitations. Hence, nonspecific activation of rumen fermentation would be of tremendous benefit. The strategies for general activation of

fermentation would involve: increased unmasking of the catalytic activity of all rumen hydrolyses and supplementation of the limiting nutrients. Limiting nutrients in silage diets will usually include the sulfur amino acids as they are susceptible to damage through peroxidation. Methionine would be broken down since it is the main precursor of ethylene biosynthesis - an extensive process that triggers senescence on harvesting herbages.

Hence research into general activation should combine factors that lead to increased unmasking with supplementation of limiting nutrients. In the feeding of forages these may include pH buffers, supplementation with sulfur amino acids in combination with Tween 80 - type surfactants.

b) *Specific nutrient activation of rumen fermentation.* In feedlots and in most intensive dairy systems, where high energy diets are fed, increased fermentation of dietary forages, particularly the structural carbohydrates, in conjunction with limited protein breakdown in the rumen is desirable. Obviously, enhancing ruminal degradation of high quality proteins would be counter-productive. Hence, the positive effects of general activation mentioned above would have to be coupled to CYS-SH inhibitors like Teepol, potassium iodate or other amenable thiol oxidants, e.g., vitamin C (dehydroascorbate). In our tests, cellulase activity was not inhibited by PCMB or 0.1% Teepol. In addition, most proteolytic bacteria were not only amylolytic, but belonged to serine and cysteine protease class. Ionization of their strongly nucleophilic active sites would be inhibited by anionic surfactants.

7.2 Fermentation strategies at ensiling

Immediately on ensiling, the primary concern is to minimize proteolysis. Direct acidification and microbial inoculation have been successful at various times. Although, inoculation with either enzymes or live lactic acid producing bacteria is less hazardous than acidification, it has a biological lag phase. It is during this period that bacterial colonization takes place. Over the same period, extensive proteolysis may take place. This possibly explains why conflicting results have been reported after silage inoculation. The nutrient specific activation mentioned above would be more amenable and requires further investigation.

The initial proteolysis that takes place in the first 3 to 5 d needs to be arrested. Our studies concurred with the fact that plant proteases have a catalytically active CYS-SH residue. At 0.1%, Tween 80 enhanced lactic acid synthesis, thus establishing a lower pH much earlier than in the control treatment. However, extensive proteolysis was still observed, though significantly less than in the control. Further, it was also concluded that SDS, Teepol and KIO_3 had the potential to reduce silage proteolysis. However, significantly less lactic acid was produced with these treatments. Silage treatment with these products may not be viable, given the application problems, unless proper application equipment is available, and possible silage instability.

The direction of appropriate interventions for the ensiling process therefore needs further clarification. At the early stages of ensiling, protease inhibitors seem appropriate. The main contributors to the proteolytic activity are plant hydrolases

triggered particularly by the hormone ethylene. Although inhibitors of ethylene biosynthesis, may seem the natural strategy, proteolysis is already in progress by the time silos are loaded. Successful protease inhibition may have to rely on the application of the additives either at harvesting or in the silos. The latter attempts to slow down a process that is already underway. An inexpensive strategy would involve protease inactivation by oxidizing agents, including gases, followed later by acidification, preferably by inoculation with lactic acid producing bacteria and cellulases. Tween 80 could be used to enhance the latter processes. Acidification is important in providing silage stability. However, the rumen pH may need to be neutralized in order to enhance ruminal fibre digestion at the time of feeding.