

Improving Alfalfa Utilization



Biochemical Strategies for Improving Alfalfa Utilization in Dairy Production

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So began the stationary journey of plants.

Introduction

From the earliest days of gathering and planting seeds man has sought to adapt plants to fulfill his needs. Today we are opening more doors and revealing the secrets that will enhance the potential use of plants. Nevertheless, we are far from a complete understanding of the relationships between structure and function during plant development. A challenge is to alter plants to maximize desirable traits and minimize undesirable ones. Attributes that are desirable in one situation may be undesirable in another depending upon the ultimate use of the plant. How do we alter plants to fit our needs without sacrificing the good qualities already expressed by a given forage? It is critical to understand the molecular and biochemical mechanisms that underlie a given function, phenotype, or process expressed by the plant. This paper describes how basic biochemical and chemical information can lead to potential strategies for improving forages. In many cases once we understand the chemistry behind a given plant trait we also know a lot about the biochemical process responsible for the trait. A progression from basic molecular/biochemical studies to the application of this information to improve forages will be presented. The first project deals with the characterization of wall pectins that lead to the development of a selection criteria for increased digestibility. The second project deals with limiting protein degradation during forage ensiling.

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Selecting legume forages with increased pectic polysaccharides

Pectin Carbohydrates

What are pectins? Why are we interested in pectins? What do they have to do with improving forages? Why can't pigs fly? Oh, sorry, that's a whole different story. Let's begin with the first question — what are pectins? The term pectin has become a common or generalized term to represent a large group of complex carbohydrates. As this group of polysaccharides is investigated in greater detail, the complexity seems to continually increase (Stephen 1983). Although there are several types of chemical structures composing this group of complex polysaccharides (including rhamnogalacturonans I, rhamnogalacturonans II, homogalacturonans, arabinogalactans, etc.), let us only concern ourselves with a generalized structure. (There are some details that only a carbohydrate chemist or biochemist can get excited about.) To put everybody on the same ground, let's build a generalized molecule to represent our pectins.

As mentioned the individual characteristics of this group of carbohydrates varies from plant to plant, but there are distinctive features that are common to most pectins. To begin construction of our molecule, we need basic building blocks (i.e., sugars, Fig. 1). These are the acidic sugar, galacturonic acid (GalA), and the neutral sugars rhamnose (Rha), arabinose (Ara), and galactose (Gal). The arrangement of the sugars varies from plant to plant. Generally, the major structural feature is regions of contiguous GalA (n= 70-100) interrupted by regions that contain a high degree of intramolecular substitution of rhamnose (Fig. 2; these can be quite extensive, in some cases being up to 300 residues of alternating Rha and GalA, (Bacic *et al.* 1988; McNeil *et al.* 1984). In practical terms this disrupts the nice linear nature of the pure GalA portion of the molecule (referred to as the homogalacturonan region, Fig. 2). In addition, the rhamnose residues are the major sites of arabinose and galactose branching (Fig. 2).

These branch regions result in some of the greatest structural complexity of pectin molecules, since substitution may vary from single sugars to highly branched polymers of Ara, Gal, or both. Pectin molecules that contain highly substituted regions are quaintly referred to as containing "hairy regions."

A second feature that is critical for pectin function is the acid group on GalA. In the wall, this acid group will be ionized producing a negative charge (Fig. 3). There are times when high levels of negative charges would be a disadvantage in the developing wall. Plants have cleverly gotten around this problem by converting the acid group to a methyl ester (Fig. 3), eliminating the negative charge. Of course plants have developed the enzymes (polygalacturonan methyl esterases) to remove the methyl ester from the acid group restoring the charge when needed in the wall matrix.

Regions of the homogalacturonans can be bound together by calcium bridges (Fig. 4). In other words Ca^{+2} ions can form ionic bonds to GalA on opposite chains of polygalacturonans and hold the two chains in a relatively fixed position. The combination of degree of substitution in the hairy regions, the degree or length of the charged regions, and the degree of esterification provide pectins with unique and variable properties (depending upon the plant and developmental stage) in aqueous environments. These properties perform critical structural functions during the development and expansion of the cell wall. If one views the molecule as a whole, there are regions that are "smooth" (polygalacturonan regions) and regions that are "hairy" (Fig 4). Pectin hairy regions can be quite extensive resulting in large three-dimensional networks that tend to entrap water and prevent close interactions with other types of molecules. The combination of pectin structural features leads to the formation of gels with a wide variety of physical properties. Although we do not understand all of the nuances of pectin properties on plant wall development, these same physicochemical properties of the pectins form the basis for many products in the food industry; e.g., thickeners, binders, jam & jelly making, etc.

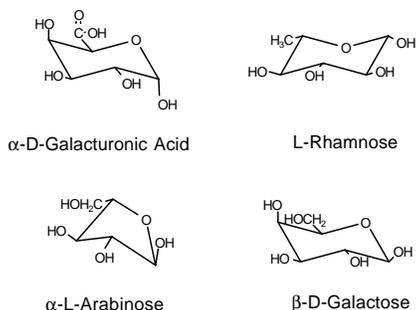


Figure 1. Major sugars in pectins.

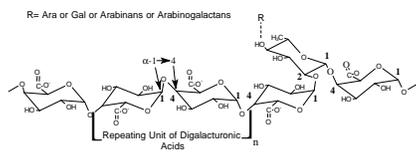
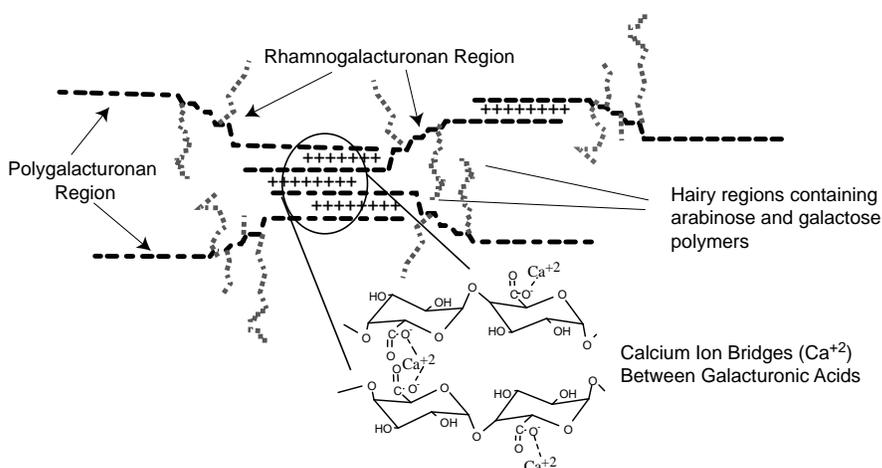


Figure 2. Major structural features and linkage patterns in a generalized pectin molecule; n=70-100



Figure 3. Removing the negative charge on pectins by forming methyl esters (neutral charge).

Figure 4. Schematic representation of pectin interactions within the wall matrix.



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“... alfalfa stems and leaves contain substantial amounts of pectins within their wall matrices (10-15% on a dry matter basis) ...”

“If we wanted to increase the digestibility of forages, adding pectins would be one way to do it.”

Pectins in alfalfa

Now that we have a feel for pectin structure and understand what makes a good jam or jelly why are we particularly interested in pectins in forages? It has been known for some time that commercial pectins are rapidly degraded by ruminal microbes (Gradel and Dehority 1972). Limited studies with alfalfa indicated that pectins appeared to be rapidly degraded (Titgemeyer *et al.* 1992) although the extent was not always apparent. When we started to investigate wall structural features that impact degradation of alfalfa stems, two observations were a bit surprising — alfalfa stems contained a significant amount of pectins (10-15%) and not all were easily solubilized using the typical pectin extraction procedures (Hatfield 1992). In fact, about 30% could not be extracted until the walls had been treated harshly enough to break covalent bonds (i.e., delignification). This posed a serious question — does the pectin in the stem degrade as quickly or as completely as assumed?

Our work showed that, although pectins isolated from alfalfa stems had complex structures (Hatfield 1991), they were rapidly and completely degraded by ruminal microbes (Fig. 5; Hatfield and Weimer 1995). There are no structural features that inhibit degradation in spite of a high degree of structural complexity. However, what about in the wall matrix? Would the 30% that was resistant to extraction also be resistant to degradation? The answer was no. Only about 10% of the pectins in highly lignified stems showed any resistance to ru-

minal microbial degradation (Fig. 6; Hatfield and Weimer 1995). More importantly, the pectins had relatively high rates of degradation out of the wall matrix (Table 1). The bottom line is that alfalfa stems and leaves contain substantial amounts of pectins within their wall matrices (10-15% on a dry matter basis) and they are rapidly and extensively degraded. Moreover, VFA production was limited to acetate and propionate and there was no downward shift in the pH even when abnormally high levels of pectins were supplied to ruminal microbes (Hatfield and Weimer 1995). Pectins appear to be an ideal carbohydrate source for ruminants. Dairy animals fed by-products that are rich in pectins from the fruit and veggie industry, do exceedingly well.

If we wanted to increase the digestibility of forages, adding pectins would be one way to do it. There are several advantages to changing forages in this manner. As already described, pectins are rapidly and extensively degraded by ruminal microbes; this rapid fermentation does not result in a pH drop in the rumen. In addition, pectins are not subject to metabolic turnover in the plant. Once the pectins have been incorporated into the wall matrix, they can be modified but not metabolized. This is not the case for cytoplasmic carbohydrates that function as building blocks (activated sugars), energy transport (sucrose), or energy storage (starch) during plant development. These sources are constantly being turned over by the plant even after harvesting. Pectins remain

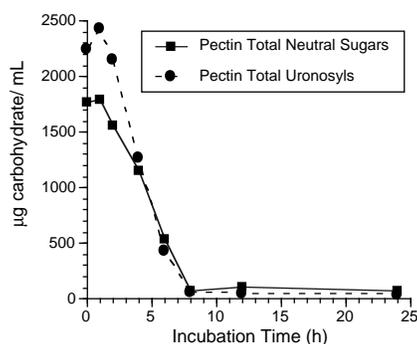


Figure 5. Degradation pattern of pectins isolated from alfalfa.

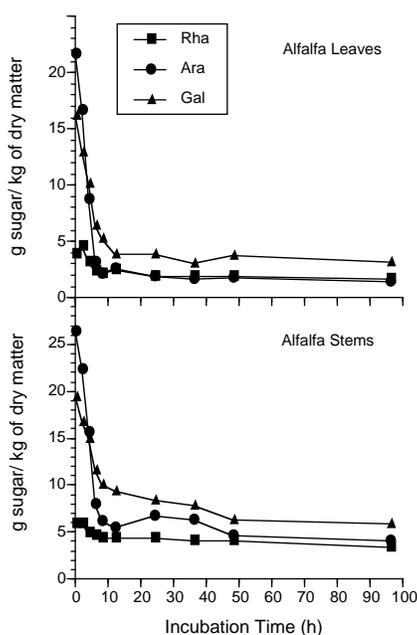


Figure 6. Degradation of pectins from alfalfa cell walls.

Table 2
Pectin content of a small sample of plants.

Plant	Leaf	Stem
	% dry matter	
Blazer	13.13	12.52
Vernal	15.02	12.75
Saranac	14.38	13.37
M. sativa A	13.79	13.93
M. sativa B	13.8	13.87
M. sativa C	13.72	13.62
Cornel Hi Pro	13.79	13.34
Can.Lo Sol Pro	14.54	13.22
Can Hi Sol Pro	14.86	13.30
M. falcata A	14.78	14.43
M. falcata B	15.56	14.67
M. falcata C	15.18	14.48
Hill Lo Lig	14.05	12.05
Hill Hi Lig	14.23	12.48
Red Clover Mar	14.60	14.48
Trefoil Witt	14.55	13.12

Table 1. Comparison of degradation rates of pectic polysaccharide components.

Sample	Degradation Rate (h ⁻¹)									
	TS	SE*	TU	SE	Ara	SE	Gal	SE	Rha	SE
Alf Pec	0.417	±0.041	0.420	±0.057	0.452	±0.068	0.612	±0.124	0.400	±0.059
CW Lvs	0.138	±0.007	0.424	±0.066	0.287	±0.112	0.255	±0.012	0.137	±0.088
CW Stm	0.044	±0.009	0.251	±0.066	0.266	±0.049	0.124	±0.018	0.096	±0.026

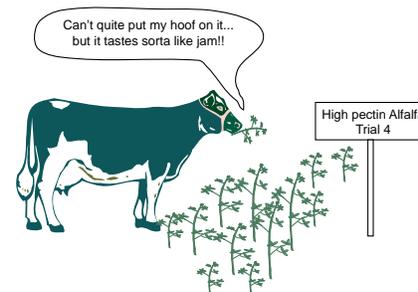
*SE = STD error of the coefficient N = 6. Degradation rates are based on the linear regression of logarithmic transformation plots. All plots were the mean of three separate experiments with eight observations per sample.

virtually unchanged from the time they are incorporated into the wall matrix to the time of feeding.

Since pectins provide a potential benefit to the animal, can we increase the amount in forages? Is there genetic diversity for this trait among alfalfa? (Realistically, legume forages are probably the only ones that have sufficient pectins to be concerned with as an adequate supply of energy. Grasses do contain pectins, but usually less than 1% of the dry matter, whereas dicots in generally have 5 to 20%). To answer this question, we developed an analysis system to determine the total pectic content of legume forages that did not rely on the extensive extraction procedures utilized in the past. What we came up with was a system that would give us accurate results in a fairly short time frame (Scheme 1. This method is accurate but too time consuming for general or large scale screening). Using this procedure, a small number of plants were evaluated for total pectin content (Hatfield and Smith 1995). Even within this relatively small group, there was a reasonable range in total pectins on a dry matter basis (Table 2). This has been encouraging and prompted us to continue development of procedures that allow rapid screening of forages to estimate total pectin content. One possibility is to simply use a total uronosyl measurement on dried samples (see Scheme 1) shortening the evaluation time considerably and allowing hundreds of samples to be processed per week. Work is also continuing along the line of developing NIRS equations to predict the levels of pectins. Other systems are also being considered as possible screening procedures.

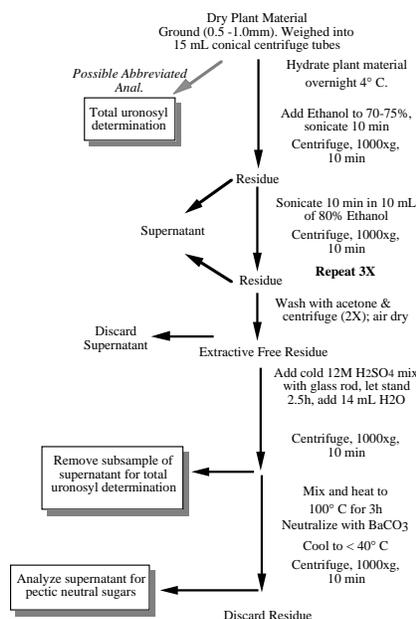
This work arose from investigations into the basic structural features of alfalfa cell wall carbohydrates and how

these structural features impact degradation. The project has grown and progressed from this basic molecular characterization of alfalfa pectins to the development of procedures that allow rapid screening of alfalfa populations for total pectin content. It seems feasible that selection could be made to increase alfalfa pectins.



Preserving plant protein during ensiling

Let's turn our attention to a problem that decreases the nutritional value of alfalfa. The problem is extensive protein degradation that frequently occurs during ensiling of alfalfa, leading to losses of up to \$70/ha through poor utilization of nitrogen by cattle (Rotz *et al.* 1993). Enzymes within alfalfa (i.e., proteases) degrade 44 to 87% of the cytoplasmic protein to ammonia, amino acids and small peptides (Muck 1987). The impact of alfalfa proteases is minimized when rapid fermentation quickly lowers the pH to a level that inactivates these enzymes. Unfortunately this does not always happen. Solutions to this problem have utilized a variety of approaches to minimize protein degradation. Some of the most effective approaches have one or more limitations including cost (heat treatment), requirement for handling caustic materials (formic acid treat-



Scheme 1. Outline of pectin analysis procedure.

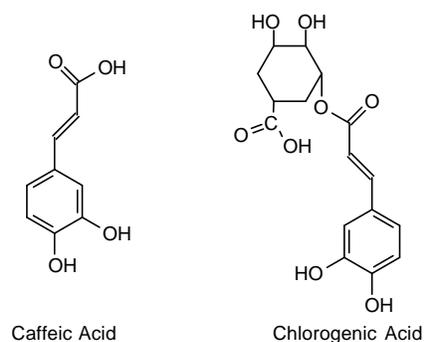


Figure 7. *o*-Diphenols commonly found in plants.

“...extensive protein degradation frequently occurs during ensiling of alfalfa, leading to losses of up to \$70/ha through poor utilization of nitrogen by cattle.”

ment), or potential health risks of the chemicals (glutaraldehyde and formaldehyde).

A couple of years ago we began to investigate red clover since it had been observed that, although the total protein levels were similar to alfalfa, there was substantially less protein degradation during the ensiling process (Jones *et al.* 1995a). Red clover would be a better legume forage for ensiling if it did not have some other undesirable agronomic traits (lower biomass yield, low stand persistency, and slow drying rate) (Holland and Kezar 1990; Smith and Kretschmer 1988; Undersander *et al.* 1992). What we were interested in was why red clover made much better silage although on outward appearance the plant did not seem to be significantly different from alfalfa. A detailed study (Jones *et al.* 1995a) was undertaken to determine if there were inherent differences in the type of proteins it contained or if there were differences in the proteases (i.e., different pH optima, lower total activity, etc.). What we found was that there were no differences in the general types of proteins present or in their protease degradability and that the protease activity and characteristics were similar to those of alfalfa. In addition, red clover does not contain tannins, as do some legume forages (birdsfoot trefoil), that can bind to proteins and prevent their degradation. So what is the critical factor in red clover?

As it turns out, red clover contains two things that are not found in any appreciable amounts in alfalfa: small soluble phenolics (e.g., caffeic acid; see Fig. 7) and a special enzyme called polyphenolic oxidase (PPO). Our work indicates that the lower proteolytic activity is due to the soluble PPO. Before I explain the experimental evidence for this conclusion, let me describe a polyphenol oxidase. This type of enzyme is found in virtually every type of plant but the amount varies greatly. Anyone who has eaten fruits or vegetables has run across this enzyme. When an apple, pear, peach, or potato (these are only a few common examples) is cut open and exposed to air, the cut surface begins to brown. The brown discoloration is the result of PPO activity. Two things are required by the enzyme — one is a phenolic substrate (preferably an *o*-diphenol; see Fig. 7) and the other is oxygen, O₂. PPO reacts with an *o*-diphenol in the presence of oxygen to form a very reactive molecule referred to as an *o*-quinone that in turn reacts with other molecules, including proteins and additional phenolics that may be in the plant, to form the brown colored material (see Fig. 8 for the molecular mechanism behind this reaction). The products formed from the PPO activity not only detract from the appearance but also can impact the taste of foods. There is a lot of effort in the food industry to eliminate or at least minimize PPO activity so as not to detract

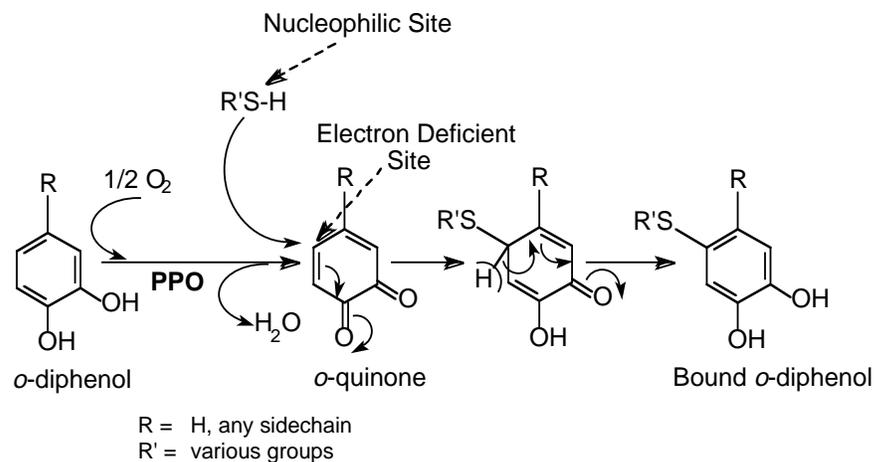
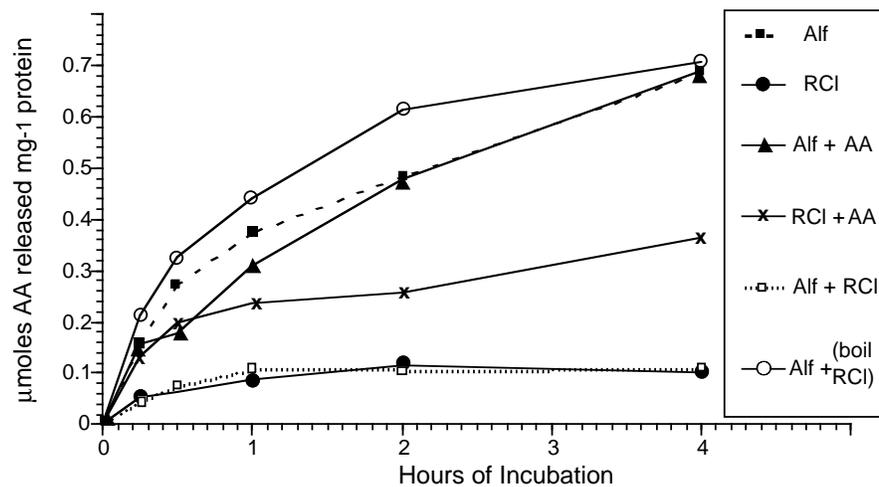


Figure 8. A generalized reaction mechanism for PPO reaction with an *o*-diphenol to form an *o*-quinone that reacts with a suitable molecule. Additional reactions can occur to produce polymers of phenols or phenols plus proteins.

Figure 9. Proteolysis of alfalfa and red clover extracts. Alf = untreated alfalfa extract; RCl =, untreated red clover extract; Alf+AA = alfalfa extract with 20 mM ascorbate and 5 mM dithiothreitol; RCl+AA = red clover with 20 mM ascorbate and 5 mM dithiothreitol; Alf+RCl = untreated alfalfa and red clover extracts mixed 1:1; Alf+(boil RCl) = untreated alfalfa and boiled red clover extracts mixed 1:1. Proteolysis was monitored by the release of free amino acids.



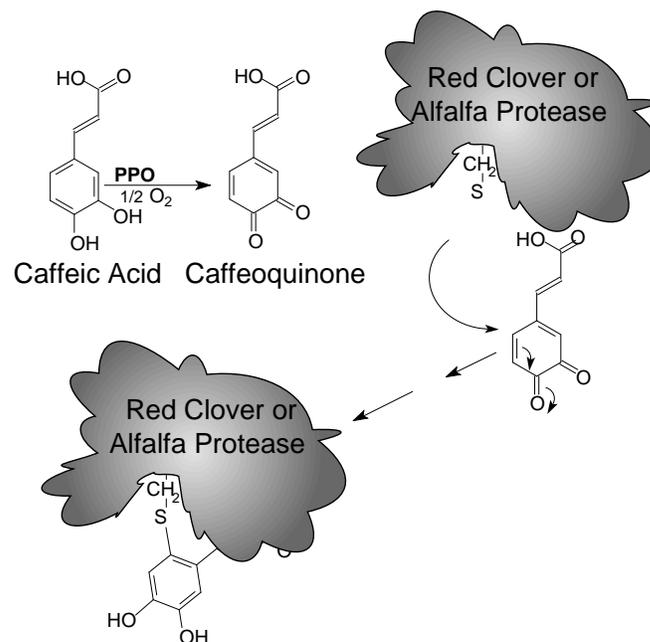
from the nutritional and aesthetic value of the foods we eat. A critical component for PPO activity is oxygen; therefore antioxidants play a big role in food preservation.

A red clover polyphenol oxidase effectively inhibits proteolysis.

Now back to our red clover story. The conclusion that PPO is involved in red clover protease inhibition is based upon the following observations (Fig. 9; Jones *et al.* 1995b): 1) Factors within red clover can rapidly reduce proteolysis in both red clover and alfalfa extracts (Fig. 9 RCl; Alf+RCl); 2) When antioxidants (such as ascorbate) were added to red clover extracts (Fig. 9 RCl+AA), proteolysis was initially similar to alfalfa (Fig. 9 Alf), but as the antioxidants were depleted, inhibition of proteolysis occurred; 3) Inhibition of alfalfa proteoly-

sis did not occur when the red clover extract was boiled to inactivate enzymes prior to mixing with alfalfa extracts (Fig. 9 Alf+boil RCl); 5) Extracts of red clover contain several types of soluble phenols including caffeic acid. During incubation of red clover extracts, there is a decrease in caffeic acid along with two other as yet unidentified phenols concomitant with the decrease in proteolytic activity. No similar loss is observed in samples containing antioxidants or in boiled controls. The inhibition of proteolytic activity in red clover is heat labile and dependent upon maintenance of an oxidative potential. Thus it is evident that the inhibition of proteolysis in both red clover and alfalfa extracts is due to the red clover PPO.

Figure 10. Possible reaction of PPO generated caffeoquinone with proteases.



“... red clover has a mechanism for inhibiting protein degradation that may be exploited in alfalfa and other forage plants that are susceptible to extensive protein degradation during ensiling.”

Although the evidence is strong that PPO and the presence of soluble phenolics are responsible for the lower protein degradation in red clover, the specific mechanism has not been established. It is most likely that the mechanism is due to the direct binding of *o*-quinones, produced by PPO, to proteases (Fig. 10). How and what amino acids are actually involved has not been established in the red clover system. There are only a few amino acids that would be nucleophilic sites for quinone attack. We are currently in the process of characterizing this mechanism. We believe that red clover has a mechanism for inhibiting protein degradation that may be exploited in alfalfa and other forage plants that are susceptible to extensive protein degradation during ensiling.

Conclusions

To develop forages that contain the attributes that are most desirable and at the same time limit those that are undesirable we must understand the plant at the chemical, molecular and biochemical mechanisms level. Increasing wall pectins for example will increase the rate of wall degradation and should provide better utilization of plant protein. By maintaining the rest of the wall intact (e.g., lignin, phenolics) there should be less danger of producing a plant that is more susceptible to environmental stresses. Also by understanding the mechanism behind the observed red clover ensiling process that preserves more plant protein, we may be able to develop treatments or modify plants that can capitalize on this mechanism.

Acknowledgment

The “we” I continually referred to in this paper reflects the inputs of several people with the USDFRC and is not just the proverbial frog in my pocket. The work reported here is the result of collaborative efforts of several people including D. Buxton, H. Jung, D. Mertens, J. Ralph, and P. Weimer of the Cell Wall Group, and R. Smith and R. Muck.

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