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Latent and Active Polyphenol Oxidase (PPO) in Red Clover (*Trifolium pratense*) and Use of a Low PPO Mutant To Study the Role of PPO in Proteolysis Reduction

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Polyphenol oxidase (PPO) activity in leaf extracts of wild type (WT) red clover and a mutant line expressing greatly reduced levels of PPO (LP red clover) has been characterized. Both latent and active forms of PPO were present, with the latent being the predominant form. PPO enzyme and substrate (phaselic acid) levels fluctuated over a growing season and were not correlated. Protease activation of latent PPO was demonstrated; however, the rate was too low to have an immediate effect following extraction. A novel, more rapid PPO activation mechanism by the enzyme's own substrate was identified. Rates of protein breakdown and amino acid release were significantly higher in LP red clover extracts compared with WT extracts, with 20 versus 6% breakdown of total protein and 1.9 versus 0.4 mg/g FW of free amino acids released over 24 h, respectively. Inclusion of ascorbic acid increased the extent of protein breakdown. Free phenol content decreased during a 24 h incubation of WT red clover extracts, whereas protein-bound phenol increased and high molecular weight protein species were formed. Inhibition of proteolysis occurred during wilting and ensilage of WT compared with LP forage (1.9 vs 5 and 17 vs 21 g/kg of DM free amino acids for 24 h wilted forage and 90 day silage, respectively). This study shows that whereas constitutive red clover PPO occurs predominantly in the latent form, this fraction can contribute to reducing protein breakdown in crude extracts and during ensilage.

KEYWORDS: Red clover; polyphenol oxidase; latent enzyme; protein degradation; silage; protein-bound phenol

INTRODUCTION

Higher plant polyphenol oxidases (PPO) are copper-containing enzymes that catalyze both hydroxylation of monophenols to *o*-diphenols and oxidation of *o*- and *p*-diphenols to *o*- and *p*-quinones (*1*). It is widely reported that these enzymes are nuclear-encoded and targeted to plastids (*2*). PPO enzymes may exist in either an active or inactive form. The inactive or latent state may be activated by a variety of treatments that alter the conformation of the enzyme, including addition of fatty acids, alcohols, and detergents and adjustment of the pH (*2*). Protease treatments can also activate the enzyme, and Jimenez and García-Carmona (*3*) concluded that access to the catalytic site is controlled by a regulatory domain/region which is either shifted by changes in conformation or removed by proteolytic cleavage. This is further supported by the work of Gerdemann et al. (*4*), who demonstrated the presence of a C-terminal extension peptide in the cDNA sequence but not in the mature form of sweet potato catechol oxidase. Because endogenous phenolic substrates are localized in vacuoles, PPO activity will occur only following activation of latent enzyme and disruption of cellular compartmentation.

Browning of fruits and vegetables during storage may be largely attributed to chemical reactions of PPO-generated quinones. Quinone products are highly reactive and can readily undergo polymerization and also bind covalently with nucleophilic sites on other phenols or amino acids in a protein chain, generating black/brown products (e.g., melanin). The resulting addition products may be further oxidized to their respective quinones, and a second addition may occur, resulting in the formation of cross-linked protein polymers (*5, 6*). It has been demonstrated that such protein−phenol complexes are resistant to enzymatic digestion by proteases including trypsin, α-chymotrypsin, and pepsin (*6, 7*). PPOs of food crops have been extensively studied as a consequence of the commercial losses associated with browning. However, until relatively recently no nutritional significance has been attributed to PPO activity in

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forage crops. It is well established that the presence of tannins reduces protein degradability in forage crops. It has now been found that red clover also shows reduced protein breakdown during both rumen fermentation (*8*) and ensilage (*9, 10*) when compared with other tannin-free forages. This has led to the proposal that this protein protection is due to high levels of active PPO enzyme (*10–13*). Evidence supporting this comes from studies by Sullivan and Hatfield (*14*), which clearly show that red clover PPO reduces hydrolysis of peptides in crude extracts. However, studies on the native state of PPO enzymes and substrate availability in red clover forage have not previously been reported. In addition, studies on the effect of PPO on proteolysis have been limited to indirect measurements of amino acid release with no direct analysis of protein and phenol fractions.

In this paper we examine levels of both active and latent PPO enzyme activity and substrate concentrations in wild-type red clover (WT) and a mutant with greatly reduced levels of PPO activity (low PPO mutant, LP) over a growing season. We identify a rapid activation mechanism for conversion of the latent form of the enzyme to the active form. We exploit the low PPO mutant to study the direct effect of PPO activity on protein and phenol fractions.

MATERIAL AND METHODS

Red Clover Grown in Controlled Environment. Red clover (*Trifolium pratense*) plants, WT and LP, were clonal lines of two plants of cv. Milvus, which were selected on the basis of high and low PPO activity as described by Lee et al. (*11*). These plants were grown in pots of John Innes no. 2 loam based compost in a glasshouse with a controlled environment. Supplementary lighting was provided by a bank of 18 Phillips Son T Agro 400 W bulbs, which were computer-regulated to supply a minimum photosynthetic photon flux density (PPDF) of 200 μmol m^{-2} s^{-1} for a 16 h photoperiod starting at dawn. Temperature was regulated to 20/15 °C during the light and dark periods, respectively. For studies on proteolysis, 10 replicate mature plants were cut to ca. 5 cm above soil level, and all fully expanded leaves were harvested and stored at −80 °C. Plants were harvested after 5 weeks of regrowth (cut to ca. 5 cm above soil level) to provide material for wilting and silage experiments.

Crosses. Hand crosses were made between five clonal plants of WT and five clonal plants of LP red clover grown as decribed above. Crosses between 10 individual F1 heterozygotes progeny were made to produce an F2 generation, and the F1 progeny were also backcrossed with WT and LP plants. All progeny were grown in a glasshouse as detailed above and screened for PPO phenotype using a rapid screen assay described below. Plants with a low PPO phenotype were grown for generation of LP seed.

Rapid Screen Phenotype Assays. For the purpose of screening for PPO phenotype, two or three leaves from each progeny plant were placed in a 2 mL microtube, flash-frozen in liquid nitrogen, and stored at −80 °C. The frozen red clover leaves were extracted in 1 mL of McIlvaine buffer (pH 7) with two large, stainless steel shot-ball cones ($^1/_4$ in.; Glen Creston) using a Retsch MM3 mixer mill set for a 1 min shake at 30 shakes s^{-1}. Extracts were then centrifuged at 15000*g* for 10 min at 4 °C, and the supernatant was transferred to a clean transparent microtube. Microtubes were vortexed, and the color of the extract was scored. A 200 μL volume of 20 mM caffeic acid in McIlvaine buffer (pH 7.0) was subsequently added to 200 μL of the extract and, after vortexing, color was again scored. Development of a brown color with or without caffeic acid indicated a WT phenotype, whereas retention of a green color under both conditions indicated an LP phenotype.

Field-Grown Red Clover. WT red clover (1.4 ha, cv. Milvus) was sown on August 31, 2004, at Trawscoed Research Farm (52° 25′ N, 4° 05′ W). The WT plot surrounded a subplot of 0.3 ha of LP red clover sown with seed generated as described above at the same time. Plots were maintained as silage pastures and, for the purpose of the present study, were sampled during the second year of growth. Approximately 200 g of WT and LP plant material (six replicates sampled at random points) was cut ca. 5 cm above soil level midmorning at 2 week intervals, between May 16 and October 5, 2005. The sward was cut back three times during this period. Fully expanded leaves were removed from plant material and either extracted immediately for determination of PPO substrate content or flash-frozen in liquid nitrogen and stored at −80 °C and subsequently sampled for PPO activity.

Proteolysis Studies. On six separate occasions leaves from five plants, grown in controlled environmental conditions, were ground in liquid nitrogen, and subsamples (ca. 0.5 g) were extracted at 0 °C in 2 mL of either McIlvaine buffer (pH 7.0) alone or McIlvaine buffer (pH 7.0) including 75 mM ascorbic acid and 0.3% polyvinylpyrrolidone to block PPO activity. Extracts were cleared by centrifugation at 15000*g* for 10 min at 4 °C. For each replicate leaf extract, 250 μL aliquots were put into four microtubes. A 250 μL volume of 20% trichloroacetic acid (TCA) and 0.4% phosphotungstic acid (PTA) were added immediately to the first tube (to precipitate protein and prevent any further PPO activity), which was then stored at 4 °C. The three remaining tubes were incubated in the dark at 30 °C for 6, 12, or 24 h, after which time a 250 μL volume of the 20% TCA−0.4% PTA solution was added to the tubes, which were subsequently stored at 4 °C. Following incubation for a minimum of 30 min at 4 °C (to ensure complete protein precipitation), all tubes were spun at 15000*g* for 10 min at 4 °C. The supernatant was retained for analysis of free amino acids and phenols, whereas the pellet was resuspended in 1.5 mL of 0.1 M NaOH and analyzed for protein content.

Amino Acid and Protein Analyses. Supernatant fractions were adjusted to neutral pH by combining 480 μL volumes with 265 μL of 1 M NaOH. These samples were then analyzed for free amino acid content according to the method of Winters et al. (*15*) and for phenols according to the method described by Veltman et al. (*16*) using tyrosine as a standard. Proteins and phenol-bound proteins were assayed using a modified Lowry procedure described by Winters and Minchin (*17*), which takes into account the variable response of *o*-diphenols with the Lowry assay and estimates the quantity of protein-bound phenol.

PPO Assays. Red clover leaf protein was extracted in McIlvaine buffer (pH 7.0) including 75 mM ascorbic acid and desalted with Bio-Gel P6DG (Bio-Rad, Hertfordshire, U.K.) as described by Winters et al. (*18*). The eluted fractions served as crude enzyme preparations in which PPO activity was analyzed spectrophotometrically (Biotech ultraspec, Pharmacia Biotech, St. Albans, Hertfordshire, U.K.) at 420 nm with methylcatechol as a substrate according to the method of Fraigner et al. (*19*) with some modifications. Standard reactions were carried out in a volume of 1.5 mL containing either 10 μL of WT red clover enzyme or 20 μL of LP red clover enzyme, 0.001 mM copper sulfate, and 10 mM methylcatechol in McIlvaine buffer (pH 7.0) alone or including 0.25% sodium dodecyl sulfate (SDS) for measurement of active and total (active plus latent) enzyme activity, respectively. It was necessary to add a larger volume of LP enzyme because of the very low levels of PPO activity in these extracts. The PPO reaction was initiated by the addition of the methylcatechol substrate, and quinone product was monitored by measuring the increase in absorption at 420 nm. Reactions were monitored over a 40–60 s period (10 readings s^{-1}), and rates were calculated from the linear phase of the curve with SWIFT II software (Pharmacia Biotech; Biochrom Ltd., Cambridge, Cambridgeshire, U.K.). For the purpose of investigating the effect of preincubation with *o*-diphenol (caffeic acid, chlorogenic acid, catechol, and methylcatechol) on PPO activity, desalted WT red clover extract was incubated with an equal volume of *o*-diphenol (10 mM) in McIlvaine buffer (pH 7.0) and the pH was adjusted to 7 when necessary. After various time intervals, 20 μL volumes were assayed for PPO activity with methylcatechol as described above.

Substrate Extraction. Fresh leaves were rapidly removed from the plant material, and 2.5 g was immersed in 25 mL of boiling 70% methanol for 10 min. The liquid volume was subsequently topped up to 25 mL with 70% methanol and left on a gel shaker for 2 h at room temperature. Supernatant was decanted, and the residual plant material was rinsed with two further 10 mL volumes of 70% methanol, which were pooled with the first fraction. Extracts were concentrated by rotary evaporation to a volume of 5 mL. A 1 mL volume was applied to a

C18 Sep-Pak cartridge (Waters). Phenols were eluted with 4 mL of methanol and analyzed by reverse phase HPLC (Waters Nova-Pak C18, 8 × 100 mm Radial-Pak cartridge) with a 35 min gradient of 0–70% methanol, 2 mL/min flow, and detected by PDA. Caffeic acid was used as a standard (peak area of absorbance at 340 nm), and phaselic acid was quantified in terms of caffeic acid equivalents.

Protein Gel Electrophoresis. To demonstrate changes in PPO isoforms, proteins were extracted at 0 °C in 2 mL of McIlvaine buffer (pH 7.0) including 75 mM ascorbic acid with and without 1 mM phenylmethanesulfonyl fluoride (PMSF). Proteins in fresh extracts and extracts incubated for 24 h at 25 °C were separated by electrophoresis on ready-cast IEF gradient gels pH 5–8 (Bio-Rad) according to the manufacturer's instructions. PPO activity was detected by incubation of gels in 10 mM L-3,4-dihydroxyphenylalanine (L-DOPA) in McIlvaine buffer (pH 7.0) with and without 0.25% SDS.

For the purpose of detecting the formation of high molecular weight protein−phenol complexes, leaf material (ca. 0.5 g) was extracted at 0 °C in 2 mL of McIlvaine buffer (pH 7.0). Extracts were centrifuged at 15000*g* for 10 min at 4 °C, and the supernatant was retained and incubated at 30 °C for up to 24 h. Samples (200 μL) were taken at various time points, and further enzymatic activity was stopped by the addition of 50 μL of loading buffer (0.25 M Tris, 0.025% mercaptoethanol, 5% SDS, 25% glycerol, pH 6.8) and subsequent boiling for 3 min. Soluble protein extracts were applied to 4–20% gradient polyacrylamide gels (Bio-Rad), and proteins were separated by electrophoresis according to the method of Laemmli (*20*).

Wilting Time Course. Plants were grown and harvested as described above. Plant material was chopped into 2 cm lengths, spread over a wooden laboratory bench, and left to wilt at 20 °C. Duplicate samples were taken after 0, 3, 6, 12, and 24 h, and approximately 25 g of plant material was freeze-dried for determination of dry matter (DM). Ground freeze-dried material was extracted and analyzed for free amino acids as described by Winters et al. (*15*).

Laboratory-Scale Silages. Plants were grown and harvested as described above. Plant material was then wilted for 24 h and chopped into 2 cm lengths, and the wilted material was ensiled following inoculation with *Lactobacillus plantarum* L57 in glass tube silos (approximately 100 g capacity) as described by Merry et al. (*21*). Silos were opened after 90 days. On opening, silages were thoroughly mixed and subsamples stored at −20 °C for subsequent analysis of lactic acid, volatile fatty acids (VFAs), ammonia N, soluble N, and free amino acids. Measurement of pH and microbial analyses were carried out on fresh samples.

Silage Analyses. Numbers of lactic acid bacteria, enterobacteria, and clostridia were determined on both fresh and ensiled herbage as described by Merry et al. (*21*). Analyses of DM, pH, lactic acid, and VFAs were carried out as described by Merry et al. (*21*). Total and soluble nitrogen levels was estimated according to the Kjeldahl method as described by Jacobs and McAllan (*22*). Free amino acids were analyzed according to the method of Winters et al. (*15*). Ammonia was analyzed in aqueous extracts as described by Cussen et al. (*23*).

Statistical Analyses. Results for PPO activation for both WT and LP red clover were compared by one-way ANOVA (REML; Genstat release 8.1: Lawes Agricultural Trust, 2005). Treatment means were compared using the Student–Newman–Keuls test.

RESULTS

Characterization of the Low PPO Mutant. Crosses of WT and LP mutant plants produced the WT phenotype in all F1 progeny and all backcrosses between F1 progeny and WT. Backcrosses between F1 progeny and LP mutants segregated 41% LP phenotype and 59% WT, whereas the F2 progeny segregated 21% LP phenotype and 79% WT.

PPO Enzyme and Substrate Levels over a Growing Season in Field-Grown WT and LP Red Clover. PPO activity and PPO substrate (phaselic acid) levels were measured in WT and LP red clover pasture (close to Aberystwyth, 52° 25′ N, 4° 05′ W) over a growing season in 2005. **Table 1** shows mean levels of active and latent enzyme activity over the sampling period.

Table 1. Mean Levels of Active and Latent Red Clover PPO Enzyme in Leaves of Field-Grown Plants over a Growing Season

	PPO activity (ΔOD/g of FW)		
red clover	active	latent	SED[a]
wild-type	105	400	38.8
mutant	6.1	19.7	2.70

[a] df = 46 ($p < 0.05$).

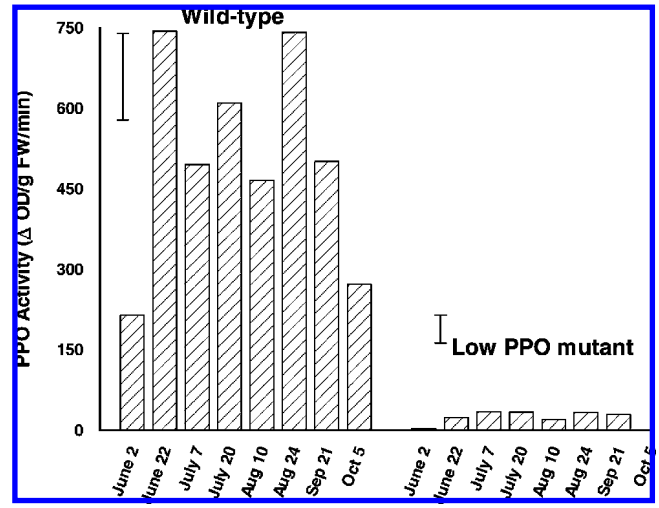

Figure 1. Seasonal variation of total PPO enzyme activity in leaves of field-grown red clover in wild-type and low PPO mutant plants. Vertical bars represent the standard error of difference (SED, $n = 3$).

Latent activity was estimated by subtracting constitutive activity (measured in the absence of SDS) from total potential activity, which was measured in the presence of SDS. Levels of latent enzyme in WT red clover were greater than those in the active form at all but one sampling time and exceeded active enzyme by an average of 3-fold, varying from 1–6.5-fold. LP plants showed greatly reduced levels of activity; however, the ratios between latent and active forms were similar to those in WT red clover (mean of 2.35:1). Whereas levels of active enzyme in WT red clover were low throughout the season, total potential activity remained relatively high despite showing some fluctuation in the same period (**Figure 1**). **Figure 2** shows phaselic acid (endogenous PPO substrate in red clover) content over the growing season in WT and LP red clover. Substrate was present at all sampling points but showed variation during this period, whereas substrate profiles did not vary significantly between WT and LP red clover ($P = 0.878$).

Characterization of Red Clover PPO Activity. Studies described above show that the latent form of PPO predominates in red clover forage; however, unpublished observations had shown that PPO in red clover extracted in the absence of ascorbic acid (a reducing agent that reverses oxidation by PPO) was largely in the active form, indicating that rapid activation occurs during preparation of crude extracts. Removal of this effect by size exclusion chromatography suggested a role for a low molecular weight compound, and phaselic acid, the major endogenous PPO substrate in red clover, was considered to be a candidate. This caffeic acid ester is not commercially available; therefore, to test the role of PPO substrate in rapid activation of the latent form, caffeic acid was incubated with a red clover PPO enzyme preparation, which was extracted in the presence of ascorbic acid and partially purified by size exclusion chromatography. PPO enzyme preparation was also incubated without caffeic acid as a control. PPO activity increased 4.7-

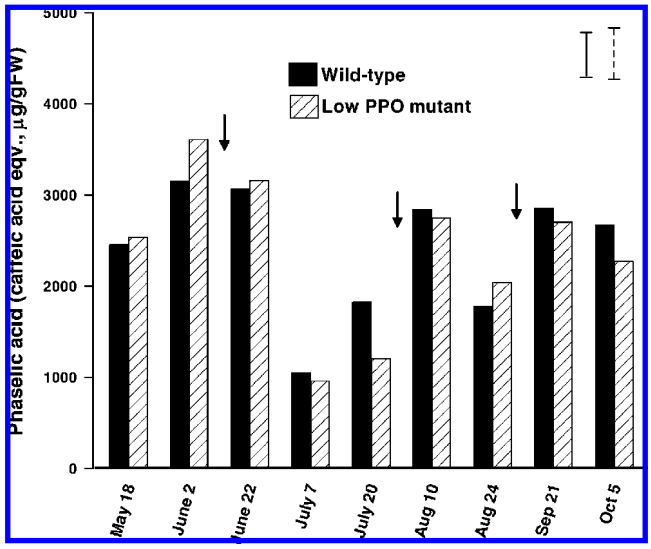

Figure 2. Seasonal variation in phaselic acid content of field-grown red clover leaves in wild-type (solid bars) and low PPO (hatched bars) mutant plants. Arrows indicate cutting times. Vertical bars represent the standard error of difference ($n = 3$) in wild-type (continuous line) and mutant (dashed line) plants.

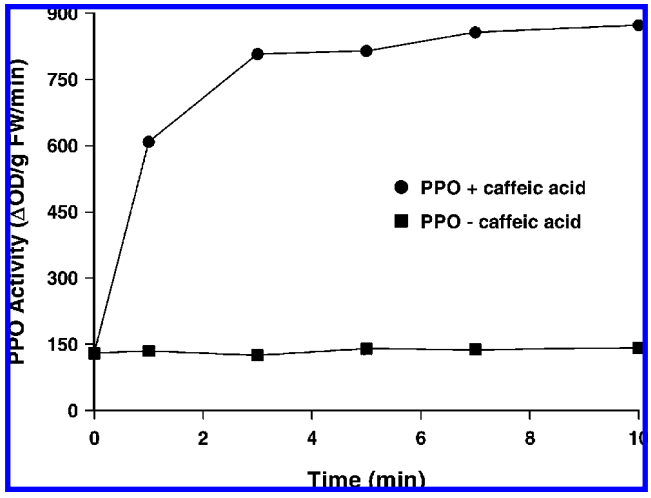

Figure 3. Levels of PPO activity in red clover extracts following incubation with (●) and without (■) 10 mM caffeic acid.

fold within 1 min and 6.7-fold after 10 min of incubation with caffeic acid, whereas no change in activity was detected in control incubations (**Figure 3**). A number of diphenols were tested for their effect on latent PPO, and all, with the exception of catechol, activated the enzyme to various extents, with caffeic acid proving to be the most effective (**Table 2**). PPO latent enzyme showed 82% activation in crude leaf extracts where PPO activity was not blocked by inclusion of ascorbic acid. Levels of activation observed following incubation of PPO with caffeic acid and an endogenous component in crude extracts were not significantly different from those observed with SDS.

To test the effect of endogenous proteases on latent PPO activity, enzyme was extracted in the presence of ascorbic acid, separated from low molecular weight compounds by size exclusion chromatography, and incubated for 20 h at 30 °C. PPO activity in this extract was compared with freshly extracted enzyme and enzyme incubated for 20 h in the presence of the serine protease inhibitor PMSF. The proportion of latent PPO activity decreased after 20 h, with a 3.9-fold increase in active PPO, whereas the extent of activation was reduced in the

Table 2. Effect of a Range of Diphenol Treatments on the Activation of Latent Red Clover PPO

treatment	% activation[a]
control	0 c
plus SDS	100 a
plus caffeic acid	91.9 a
plus chlorogenic acid	33.1 b
plus catechol	−1.6 c
plus methylcatechol	23.0 bc
extract without inhibitor[b]	82.3 a
SED	6.97

[a] Results are expressed as a percentage of the activation observed with SDS (assumed to be 100%). Means with different letters are significantly different ($p < 0.05$), $n = 3$, df = 14. [b] Leaves were extracted into neutral buffer without ascorbic acid, desalted, and assayed for PPO activity.

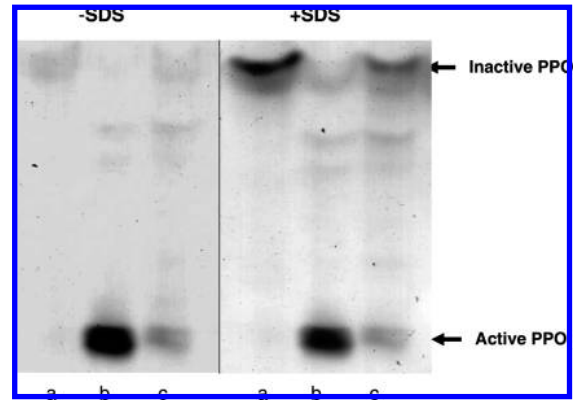

Figure 4. PPO activity following separation of red clover leaf protein by IEF gel electrophoresis of fresh extracts (lane a) and following incubation with endogenous proteases alone (lane b) or with protease inhibitors (lane c).

presence of PMSF, with only a 1.7-fold increase in active PPO. PPO isoforms in these extracts were visualized after separation by IEF gel electrophoresis and incubation with L-DOPA (**Figure 4**). Latent activity was visualized by the inclusion of SDS. One to two latent isoforms were clearly evident in the fresh extracts, but active isoforms were barely visible. Incubation resulted in almost complete disappearance of the latent isoforms with the concomitant appearance of a major active isoform. Latent isoforms persisted, and formation of active PPO was greatly reduced in extracts incubated in the presence of the protease inhibitor PMSF.

Effect of PPO on Proteolysis in Red Clover Extracts. Changes in protein and free amino acid content were monitored during incubation of WT and LP red clover leaf extracts at 30 °C for 24 h (**Figures 5** and **6**) to study the effect of PPO on protein breakdown. Both LP extracts and WT extracts in which PPO activity had been blocked with ascorbic acid showed extensive protein breakdown with concomitant increases in free amino acid content. Indeed, LP extracts with ascorbic acid showed markedly greater protein breakdown after 24 h compared with all other treatments, whereas free amino acid content was higher at all sampling times, which was significant in 24 h samples ($P > 0.05$). Conversely, the extents of protein breakdown and free amino acid accumulation were greatly reduced in WT extracts from which ascorbic acid was omitted. The degree of phenol binding to protein (protein-bound phenol) showed a negative relationship with protein degradation parameters; highest levels were observed in WT extracts and lowest levels in LP extracts. Plotting changes in bound and soluble phenol with time in WT and LP extracts without ascorbic

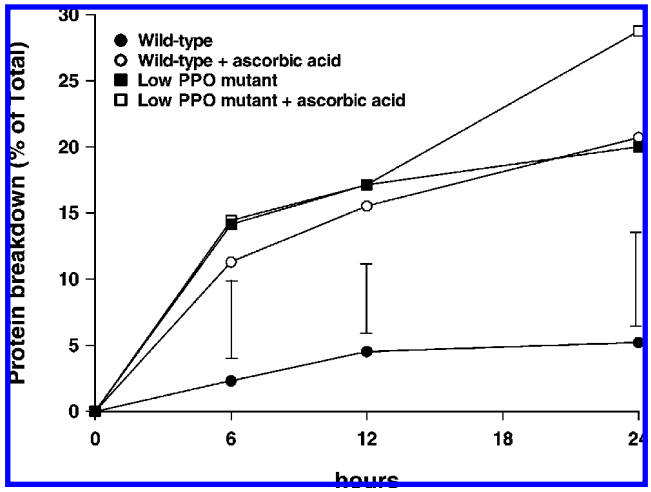

Figure 5. Protein breakdown during incubation of wild type (●) and low PPO mutant (■) red clover leaf extracts. Extracts were incubated in the presence of ascorbic acid (open symbols) and in the absence of ascorbic acid (solid symbols). Initial protein contents of wild type and low PPO mutant extracts were 22.1 and 24.3 mg/g of FW, respectively. Vertical bars represent the standard error of difference ($n = 6$) ($p < 0.05$).

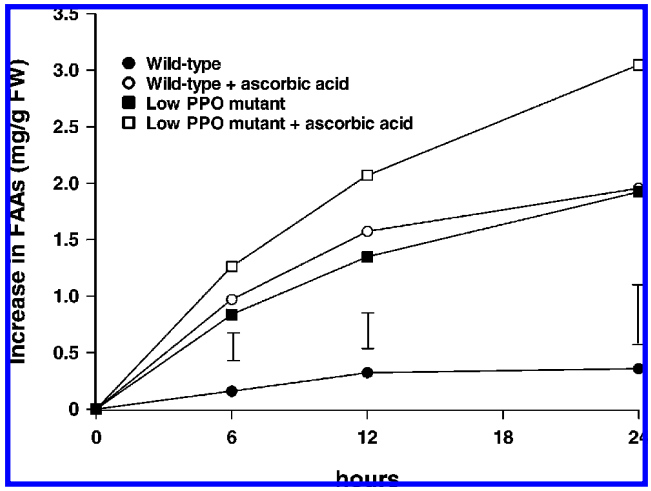

Figure 6. Increase in free amino acids (FAAs) during incubation of wild type (●) and low PPO mutant (■) red clover leaf extracts. Extracts were incubated in the presence of ascorbic acid (open symbols) and in the absence of ascorbic acid (solid symbols). Initial FAA contents of wild type and low PPO mutant extracts were 1.51 and 1.31 mg/g of FW, respectively.Vertical bars represent the standard error of difference ($n = 6$) ($p < 0.05$).

acid (**Figure 7**) showed a concomitant decrease in soluble phenol with increasing bound phenol. However, there is a discrepancy between total soluble plus bound phenol at 0 and 24 h in WT extracts (1.98 vs 1.42 mg g of FW^{-1}, respectively). This is probably due to some binding of oxidized phenol products (quinones) to nucleophilic sites of nonprotein molecules such as other phenols and nucleic acids. Analysis by SDS-PAGE (**Figure 8a**) clearly shows the rapid formation (within 3 min) of high molecular weight protein−phenol complexes in WT but not LP extracts. There is a marked change in the protein profile of WT extracts over the 24 h period with an obvious decrease in low molecular weight proteins and a concomitant increase in high molecular weight complexes (**Figure 8b**). By 24 h the de novo appearance of high molecular complexes can also be observed in LP extracts.

Pre-ensilage Wilting and Silage Characterization. In a preliminary wilting trial, free amino acids accumulated more

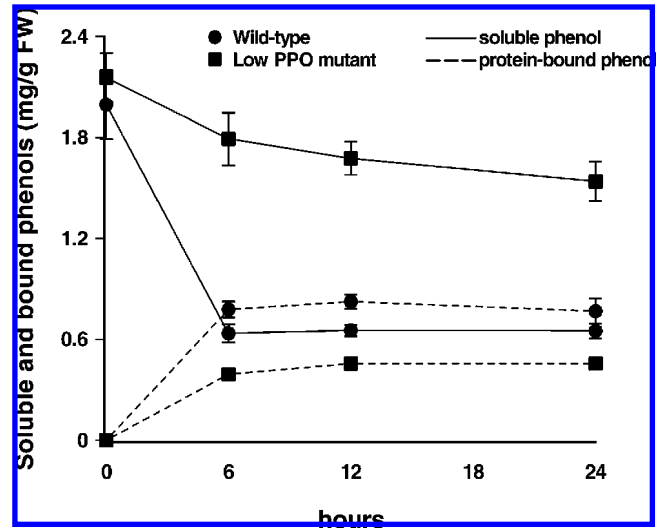

Figure 7. Changes in soluble (—) and protein-bound phenol (---) during incubation of wild type (●) and low PPO mutant (■) red clover leaf extracts. Units are based on tyrosine equivalents. Vertical bars represent the standard error of difference ($n = 6$) ($p < 0.05$).

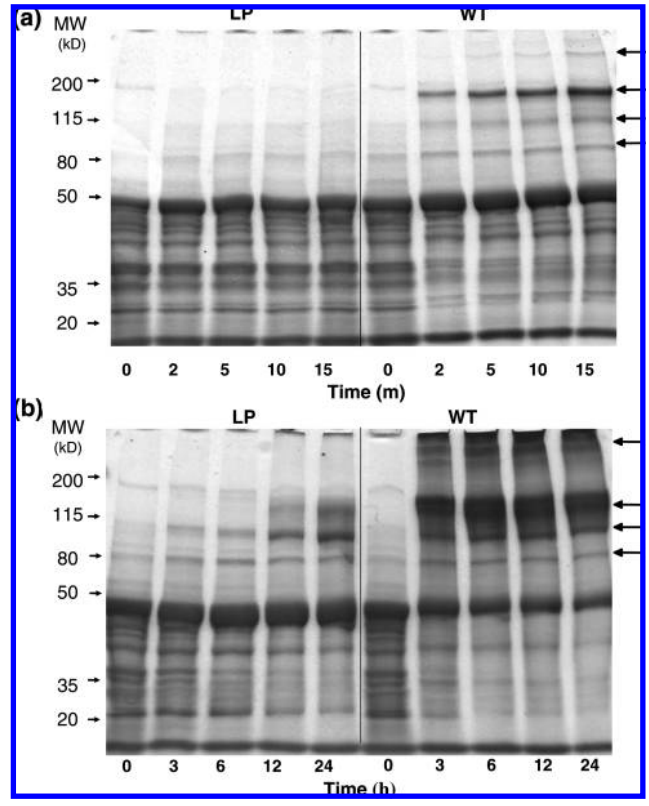

Figure 8. Separation of soluble protein by SDS-PAGE following incubation of wild type and low PPO mutant red clover extracts for (**a**) 0–15 min and (**b**) 0–24 h. Arrows indicate newly formed protein−phenol complexes.

rapidly and to a greater extent during the wilting of LP herbage (**Figure 9**). The compositions of both wilted WT and LP red clover herbage prior to ensilage were similar with the exception that LP herbage wilted to a greater extent (**Table 3**). It is unclear why the forages wilted to different extents because both had similar initial DM contents (ca. 180 g kg of FM^{-1}) and were at a similar growth stage (midflowering) when harvested. Differences in the DM content of the pre-ensiled material were reflected in silage DM contents (**Table 4**). Both forages produced well-preserved lactate silages of low pH. The lower

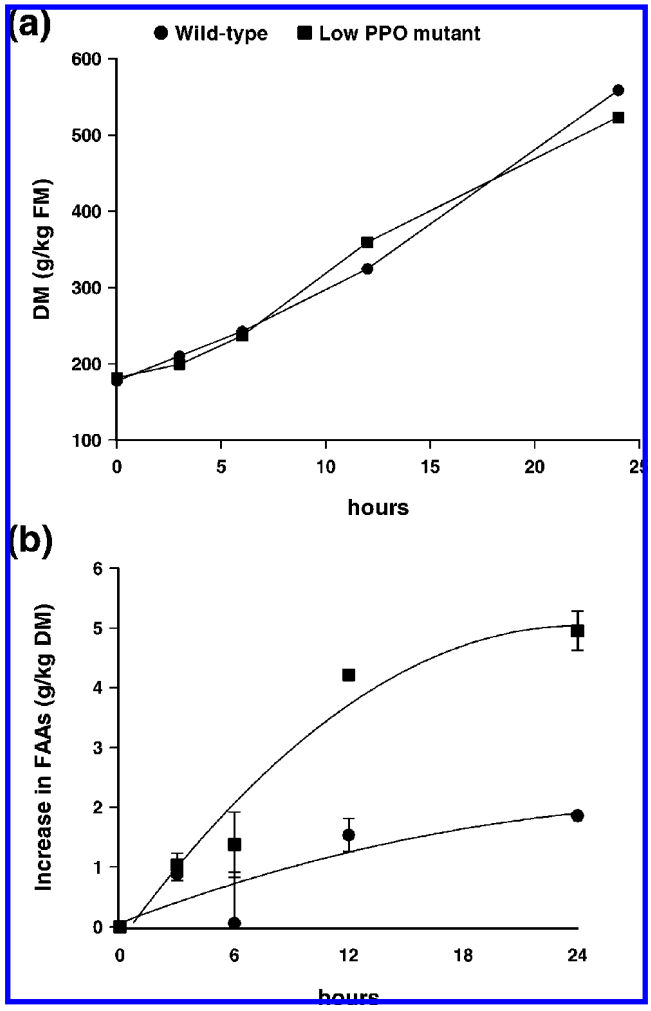

Figure 9. Increase in (**a**) DM and (**b**) free amino acids (FAAs) during wilting of harvested wild type (■) and low PPO mutant (●) red clover plant material. Vertical bars represent the standard error of the mean.

Table 3. Composition of Wild Type and Mutant Red Clover Herbage

	red clover herbage			
	wild type	mutant	SED[a]	significance
DM (g/kg of FM)	400.6	492.4	5.90	<0.001
WSC (g/kg of DM)	163.9	148.6	7.28	NS
total N (g/kg of DM)	27.9	28.4	1.44	NS
free amino acids (g of N/kg of TN)	32.6	40.4	3.91	NS
ammonia (g of N/kg of TN)	4.6	5.6	0.41	NS
buffering capacity (Mequiv/kg of DM)	500.5	570.5	25.60	NS

[a] df = 4.

pH values ($P < 0.001$) and higher lactic acid values (NS) of WT silages may indicate a less restricted fermentation due to the lower DM content of the ingoing forage. In general, the extent of protein degradation was low during ensilage of both forages; however, free amino acid and nonprotein N values for LP silages exceeded those for WT silages by 20 and 40%, respectively, indicating greater protein breakdown.

DISCUSSION

Red clover is an outbreeder, and there is a high level of genetic diversity in the Milvus cultivar. Progeny with WT and LP phenotypes were indistinguishable from each other with respect to other obvious phenotypic features, showing it is possible to have greatly reduced levels of PPO without any other obvious effects on phenotypic traits. However, PPO levels may influence less obvious traits such as resistance to specific pests or pathogens that were not encountered by red clover plants used in these studies.

Table 4. Composition of Wild Type and Mutant Red Clover Silage

	red clover herbage			
	wild type	mutant	SED[a]	significance
DM (g/kg of FM)	382.0	463.8	12.94	<0.001
pH	3.87	4.01	0.008	<0.001
lactic acid (g/kg of DM)	81.2	67.2	7.07	NS
acetic acid (g/kg of DM)	16.8	20.5	1.67	0.042
total N (g/kg of DM)	22.5	22.3	0.60	NS
free amino acids (g/ kg of DM)	17.3	21.0	0.96	0.002
free amino acids (g of N/kg of TN)	93.4	111.3	5.48	0.005
nonprotein N (g of N/kg of TN)	125.7	177.7	13.51	0.002
ammonia (g of N/kg of TN)	19.25	23.4	1.49	0.013

[a] df = 18.

In this study, constitutive red clover PPO was always present in both the active and latent form, with the latter form predominating. Levels of active enzyme in field-grown red clover were approximately 10-fold higher than levels reported in red clover grown in a controlled environment (*11*). Considerable variation in the ratios of active to latent enzyme was observed in the present study. In addition, field-grown plants may have greater exposure to pathogens and abiotic stresses compared with plants grown in a controlled environment, resulting in greater activation of endogenous latent PPO. Total levels of PPO activity in field-grown red clover showed seasonal fluctuations; however, as this study was carried out over only one season, it cannot be ruled out that variation was due to environmental effects. This is supported by the finding that PPO levels in September 2004 were approximately 5-fold lower than levels in the equivalent month in 2005 (*24*) and levels in May 2006 were almost 2-fold higher than those in May 2005 (unpublished observations). It is notable that red clover growth rates were high in the mild, wet conditions of September 2004 compared with the much lower rates observed in the drier conditions of 2005.

The major red clover PPO substrate is the caffeic−malic ester, phaselic acid, and levels observed over a growing season (equivalent to 5–20 μmol of caffeic acid g^{-1} of FW) are in good agreement with the range of 10–15 μmol g^{-1} of FW predicted by Sullivan and Hatfield (*14*). The caffeic amide, *trans*-caffeoyl L-DOPA (clovamide) was also observed but at much lower concentrations with values equivalent to 1–4 μmol of caffeic acid g^{-1} of FW (unpublished observations). There was no clear relationship between enzyme and substrate levels, and this, coupled with the observation of phenotypically normal levels of phaselic acid in LP red clover, would indicate that PPO expression is independent of substrate accumulation.

The role of PPO in ruminant forage nutrition is dependent on the presence of functional enzyme in the harvested forage. The proportion of PPO in the active form in fresh red clover herbage can be as low as ca. 10%. Latent PPO is inactive at neutral pH, and homogenized red clover forage pH is approximately 6.5 (*12*), indicating that PPO in this form will be largely inactive following harvesting. This study provides compelling evidence for an activation mechanism involving the endogenous PPO substrate following breakdown of subcellular compartmentation. The observation that the *o*-diphenols caffeic acid and chlorogenic acid are potent "activators" of latent PPO coupled with the rapid activation in extracts made in the absence of PPO inhibitor (ascorbic acid) supports this hypothesis.

Although this study clearly shows that proteolytic cleavage also results in activation of red clover PPO, this occurs at a much slower rate and cannot account for the rapid conversion of latent to active PPO observed during extraction of enzyme. It is proposed that the *o*-diphenol-mediated activation mechanism involves PPO-catalyzed oxidation of *o*-diphenol followed by interaction of quinone products with the latent form of PPO. We hypothesize that this results in altered conformation of the PPO protein, conferring activity in a similar manner to SDS treatment (*3*).

This study confirms that red clover PPO inhibits proteolysis in crude leaf extracts irrespective of the low levels of constitutive PPO occurring in the active form. This is in agreement with previous studies by Sullivan and Hatfield (*14*).

In the present study it can be concluded that conversion of latent to active form will have occurred when ascorbic acid was not included in the extraction medium. The highest levels of PPO activity would be anticipated in WT extracts and the lowest levels in LP extracts with ascorbic acid and this is consistent with observed levels of protein breakdown. Ascorbic acid is oxidized by PPO and, although initially effective when included in WT extracts, its potency may decrease with time. In addition, PPO activity is not completely absent in LP red clover extracts. These factors probably explain the intermediate levels of protein breakdown observed with these two treatments. Changes in FAA levels showed a similar pattern with upper and lower extremes observed in LP extracts with ascorbic acid and WT extracts, respectively, with intermediate levels in the other extracts. The relationship between protein-bound phenol and protein degradation parameters is convincing evidence for a link between PPO activity and protein degradation, as originally proposed by Jones et al. (*9*). The appearance of high molecular weight protein species in the presence of PPO may be attributed to the formation of protein−phenol complexes similar to those observed by Kroll et al. (*6*) in studies on the interactions of quinones with myoglobin. Previous studies (*6, 7*) also suggest that these protein−phenol complexes are resistant to proteolytic attack.

Water-soluble carbohydrate levels in red clover are inherently low. and it is common practice to wilt forage prior to ensiling to ensure a satisfactory lactic acid fermentation. Blackening of red clover leaves frequently occurs during wilting and is evidence of tissue damage and the occurrence of PPO activity. Other studies have demonstrated proteolytic degradation during wilting of forage (*25–27*), and it has also been noted that rates of protein breakdown in red clover were lower compared with other forages (*25, 26, 28*). The low levels of protein breakdown observed in silages described here may possibly be attributed to the high initial DM contents of the ingoing wilted herbage, in particular with LP forage. Previous studies have also demonstrated a relationship between the extent of protein degradation during ensilage and DM content (*29, 30*), and MacPherson (*31*) estimated that proteolysis was greatly reduced during ensilage when a DM exceeding 40–45% is attained. The high DM content of the wilted LP herbage used in these studies (49.2%) may have restricted proteolytic activity during ensilage of this material. Nevertheless, these data are consistent with previous findings (*14*) and indicate that PPO activity can have an inhibitory effect on proteolysis during ensilage even under highly restrictive conditions for protein breakdown to occur.

In conclusion, PPO enzyme and substrate are constitutively expressed in red clover but levels are variable. PPO is predominantly expressed in the latent form; however, an activation mechanism involving PPO substrate ensures rapid conversion to the active form following breakdown of subcellular compartmentation. Further studies are required to further elucidate this activation mechanism. The use of a low PPO mutant line of red clover confirms that PPO activity is responsible for the low levels of protein degradation observed in red clover extracts and silage and that this is possibly due to the formation of protein−phenol complexes which are resistant to proteolytic attack. It is difficult on the basis of this study to predict an optimal stage for harvesting to ensure maximum PPO content. There may be scope for selection of high PPO red clover varieties that are less responsive to seasonal/environmental conditions.

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