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Introduction Preserving high quality forage in cool humid regions of agricultural production remains a challenge due to potentially high levels of protein degradation during ensiling. Red clover is an exception maintaining its high protein levels during ensiling. Decreased proteolytic activity in red clover is due to polyphenol oxidase (PPO) activity and appropriate *o*-diphenol substrates (Jones *et al*., 1995, Sullivan *et al*., 2004). This work highlights potential strategies for utilising PPO as a means of decreasing proteolytic degradation during the ensiling of lucerne and other forages.

Methods Three red clover PPO genes (PPO1, PPO2 and PPO3) were cloned from a leaf cDNA library. Red clover PPO cDNAs under control of a constitutive promoter were expressed in lucerne. Leaves from PPO transformed lucerne were ground in liquid nitrogen and extracted with 50mM MES buffer (pH 6.5). Extracts were allowed to incubate at 37°C and subsamples removed at time intervals to determine the extent of proteolysis. Lucerne extracts were also incubated with or without the addition of *o*-diphenols to determine the impact of PPO activity and type of *o*-diphenol on proteolysis.

Results and discussion Since lucerne does not produce *o*-diphenols, PPO-transformed lucerne (PPO-Luc) is an excellent system for defining factors critical for PPO-mediated proteolytic inhibition. The amount of PPO activity measured in transformed lucerne (PPO-Luc) was at levels similar to that found in red clover using the *o*-diphenol caffeic acid as the primary PPO substrate. Addition of caffeic acid to PPO-Luc extracts resulted in approximately 80% inhibition of proteolytic activity (Table 1). Caffeic acid and chlorogenic acid *o*-diphenols were the most rapidly utilised substrates with other *o*-diphenols showing considerably less activity. However, a wide range of *o*-diphenols were effective in decreasing proteolysis over incubation times of 4 h or longer (Table 2). Addition of PPO-Luc extracts to oat leaf extracts along with caffeic acid resulted in a 50% reduction in proteolysis. Preliminary experiments ensiling PPO-Luc in mini-silos with the addition of *o*-diphenols resulted in a 25% decrease in proteolysis over control silos. These results indicate that strategies can be developed to utilise the PPO system to decrease protein losses during ensiling of forges.

Table 1 Amino acids released (mmol/mg total protein) from lucerne extracts incubated with or without 3 mM caffeic acid (+CA, -CA). Extracts were from Control-Luc (lucerne transformed with vector only) or from PPO-Luc (lucerne transformed with red clover PPO 1 gene)

Incubation hour	Control-Luc-CA	Control-Luc+CA	PPO-Luc-CA	PPO-Luc+CA
0	0.00±0.00	0.00±0.00	0.00±0.0	0.00±0.00
1	0.18±0.02	0.21±0.00	0.22±0.03	0.06±0.01
2	0.36±0.03	0.39±0.02	0.40±0.03	0.11±0.01
3	0.51±0.03	0.56±0.01	0.54±0.06	0.10±0.02
4	0.66±0.04	0.70±0.02	0.68±0.07	0.15±0.02

Table 2 Impact of different *o*-diphenols (3 mM) on proteolytic inhibition in lucerne extracts. Amino acids released after 4 h incubation at 30°C. CA= caffeic acid, HCA= hydrocaffeic acid, CGA= chlorogenic acid

	Amino Acid Released (mM/mg protein)				
Substrate	Luc-Control	Luc-PPO	Substrate	Luc-Control	Luc-PPO
None	0.71±0.05	0.72±0.12	CGA	0.80±0.06	0.26±0.06
CA	0.75±0.08	0.12±0.03	Catechol	0.71±0.10	0.37±0.05
HCA	0.70±0.07	0.25±0.07	Epicatichin	0.64±0.08	0.25±0.08

References

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